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# EFFECT OF DIFFERENT FORMS OF IODINE ON LAYING HENS

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FOUR FIGURES

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The seasonal variation in the iodine content of the thyroid of birds and mammals has been well established. Recently much interest has been aroused by the discovery that the iodine content of eggs can be influenced by the diet of the hen. According to Wilder, Bethke and Record ('33) the amount of iodine in the egg depends on the amount of iodine in the diet but is not influenced by the form in which it is fed to the bird.

Obviously, the feeding of iodine to laying hens raises a number of questions regarding the effect of different forms and amounts of iodine on the birds, their eggs and their progeny. In order to answer some of these questions, a series of experiments has been conducted. This paper presents the results of some of these experiments designed to determine the effects of different amounts of iodine and of iodine from different sources on laying hens.

In a preliminary experiment the birds were divided into four lots of which three lots, each comprising four laying hens, were given by mouth the following: 1, desiccated kelp (two capsules containing 815  $\gamma$  of iodine a day); 2, desiccated thyroid (one capsule containing 815  $\gamma$  of iodine a day) and 3, potassium iodide solution (2 cc. containing 815  $\gamma$  of iodine

<sup>1</sup> We are indebted to Dr. J. W. Givens for many of the iodine analyses reported in this paper.



a day). In the case of lot 4, potassium iodide solution was injected subcutaneously into two hens. The birds fed desiccated thyroid lost weight and stopped laying. The two hens into which potassium iodide solution was injected also lost weight, but continued to lay. The kelp and the potassium iodide solution (given by mouth) apparently had no effect on body weight or egg production.

To test further the effect of iodine from different sources on laying hens a ration was made up of ground yellow corn 48 pounds, ground wheat 24 pounds, yeast 2 pounds, alfalfa meal 5 pounds, dried skim milk 10 pounds, meat scrap 5 pounds, ground limestone (calcite) 5 pounds, salt 0.5 pounds, and sardine oil 0.5 pounds. This ration contained 50  $\gamma$  of iodine in 100 gm. of the ration.

Thirty-five hens in their second laying year were divided into five lots. The number of hens in each lot together with the amount of iodine fed in addition to the iodine in the basal ration is shown in table 1. Lot 1 received the basal ration only (iodine content 50  $\gamma$  per 100 gm. of feed). For lot 2 oyster shell was substituted for the calcite in the basal ration which increased the iodine content to 250  $\gamma$  per 100 gm. of feed. Lot 3 received the basal ration plus 800  $\gamma$  of iodine in the form of desiccated thyroid fed by capsule to each bird daily. The desiccated thyroid fed the first 3 weeks contained 0.308 % iodine while that fed from the fourth to eleventh weeks, inclusive contained 0.174 % iodine. Lot 4 received the basal ration plus 2 cc. daily of an aqueous iodo-salicylic acid solution containing 800  $\gamma$  of iodine. At the end of the sixth week, the amount of iodo-salicylic acid solution was doubled. Lot 5 received the basal ration plus 2 cc. daily of an aqueous sodium iodide solution containing 8000  $\gamma$  of iodine for the first 6 weeks and 4 cc. (16,000  $\gamma$  of iodine) daily for the remaining 5 weeks. The iodo-salicylic acid and sodium iodide solutions were introduced into the crop by pipette. All analyses were made by the method described by Almquist and Givens ('33).

TABLE 1

*Iodine in the yolk and albumen of eggs in  $\gamma$  per egg*

EXPERIMENT NO.	SOURCE OF IODINE OTHER THAN THE BASAL RATION	NUMBER OF HENS	AMOUNT OF ADDITIONAL IODINE GIVEN THE HENS DAILY, $\gamma$	EXPERIMENTAL PERIOD (NUMBER OF WEEKS)										
				1	2	3	4	5	6	7	8	9	11	
2	None	11		$\leq 3$	$\leq 3$	$\leq 3$	$\leq 3$	$\leq 3$	$\leq 3$	$\leq 3$	$\leq 3$	$\leq 3$	$\leq 3$	
2	Oyster shell <sup>a</sup>	12		$\leq 3$	38	35	42	42	42	42	35	39	42	
2	Decalcified thyroid <sup>b</sup>	4	800	$\leq 3$	145	....	....	400	126	116	74	830	85	
2	Iodo-salicylic acid	4	800 <sup>c</sup>	$\leq 3$	$\leq 3$	$\leq 3$	10	61	125	180	420	50	40	
2	Sodium iodide	4	8000 <sup>c</sup>	300	1190	1520	1510	1730	1300	1050	1512	...	1350	
3	Iodo-salicylic acid	4	800	1 to 4 weeks	5 to 8 weeks	9 to 10 weeks	11 to 12 weeks	13 to 16 weeks	17 to 20 weeks					
3	Sodium iodide	4	800	19.0	21.1	25.4	102.0	4.7	10.0					
				54.8	44.3	44.5	70.0	11.7	94.9					
4	None	4	See text	6th week	8th week	11th week	14th week	17th week	25th week					
4	Potassium iodide	4		10	10	10	15	20	10					
4	Potassium iodate	4		125	125	85	120	120	120					
4	Iodo-salicylic acid	4		130	105	120	130	100	100					
4	Di-iodotyrosine	4		30	10	30	...	20	10					
4	Iodized olive oil	4		30	105	80	125	100	100					
4	Decalcified thyroid (0.3 % I <sub>2</sub> )	4		25	75	50	30	30	30					
				30	65	35	50	30	...					

<sup>1</sup> Five pounds of oyster shell was substituted for the calcite in the basal ration.<sup>2</sup> See text.<sup>3</sup> Amount doubled at the end of 6 weeks.

In the third experiment, two groups of four hens each were given the same basal ration. Lot 1 received 1 cc. (800  $\gamma$  of iodine) of iodo-salicylic acid, lot 2, 1 cc. (800  $\gamma$  of iodine) of sodium iodide. Since the amount of iodine fed was small, only the iodine analysis of eggs, excreta and glands will be considered. In a fourth experiment seven groups of four hens were used. The same ration was fed to each group. One group received this ration only while the other six groups were given the iodine preparations shown in table 1 in such amount as to add 14,000  $\gamma$  to each kilogram of feed.

#### EXPERIMENTAL RESULTS

The fluctuations in the average weight of the birds in experiment 2 are shown in figure 1, the per cent egg production in figure 2 and the feed consumed by three of the five groups in figure 3. In general, feed consumption varied with changes in egg production and body weight. There was relatively little variation in the case of the birds fed the unsupplemented basal and oyster shell rations, although body weight decreased temporarily at the end of the third week. The birds given iodo-salicylic acid likewise varied relatively little after an initial decrease in weight and a temporary decrease after the amount of iodo-salicylic acid was increased at the end of the sixth week.

The birds fed desiccated thyroid ate very little the first 3 weeks. They lost weight rapidly and stopped laying. After changing the desiccated thyroid preparation fed, about the end of the third week, the birds consumed more feed, gained in weight and started to lay despite the fact that their iodine intake was the same as before. It should be noted that in this case the preparation with the higher percentage of iodine was more active per unit of iodine.

After a period of a little over 1 week, the birds fed sodium iodide decreased in weight. Egg production was apparently not affected, but when the amount of sodium iodide was doubled at the end of the sixth week, both body weight and egg production decreased. Feed consumption decreased the

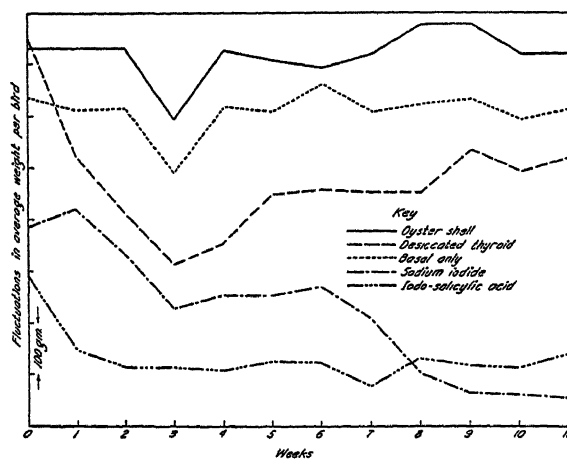


Fig. 1 Fluctuations in average weight (in grams) per bird.

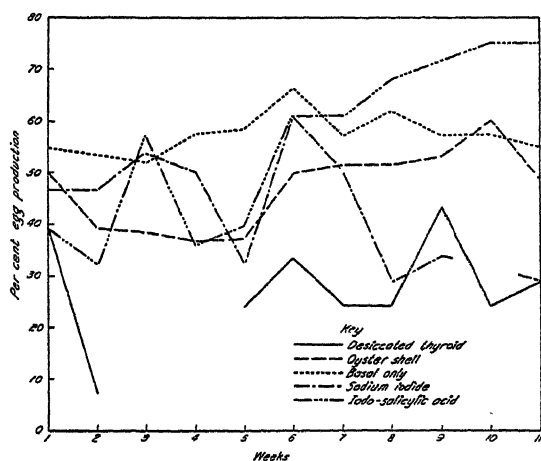


Fig. 2 Variation in the average per cent egg production of birds given iodine from different sources.

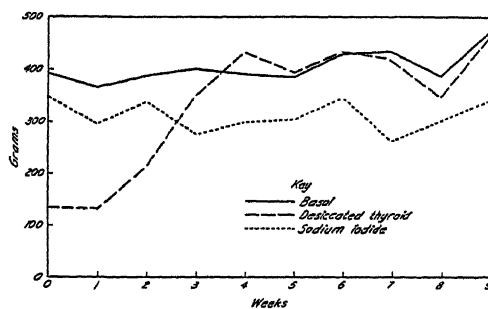


Fig. 3 Amount of feed consumed (in grams) each week per kilo of body weight.

first 4 weeks and was low the last 4 weeks (figs. 1, 2 and 3). The average weekly feed consumption per kilo of body weight for the different groups was as follows: Basal group 402 gm.; oyster shell group 349 gm.; desiccated thyroid group 324 gm.; iodo-salicylic acid group 375 gm.; sodium iodide group 308 gm.

In experiment 4 the birds fed desiccated thyroid lost weight while the body weight of the birds in other groups, with the possible exception of the birds given iodo-salicylic acid, did not change significantly. The average egg production of the various groups was as follows: No supplement, 91 eggs; potassium iodide, 113 eggs; potassium iodate, 103 (128) eggs; iodo-salicylic acid, 69 (89) eggs; di-iodotryosine, 133 eggs; iodized olive oil, 111 eggs; desiccated thyroid, 34 (44) eggs. The figures in brackets indicate the average egg production of the birds that survived to the end of the experiment. Only in the case of the birds fed desiccated thyroid did the egg production differ significantly from that of the controls.

#### IODINE EXCRETION

In experiment 2 a composite sample of six eggs selected at random from those laid on Friday, Saturday and Sunday of each week except the tenth, was analyzed for the iodine content of the mixed yolks and albumen. All the eggs laid by the birds fed desiccated thyroid were used because of the small number obtained. In experiments 3 and 4 a composite sample was taken for analysis at 2- to 8-week intervals.

The average iodine content of the eggs laid by the various groups is shown in table 1. The points of interest in this table (experiment 2) are: The consistently low iodine content of the eggs laid by the birds fed the basal ration only; the higher iodine content after the first few days of the eggs from the birds fed oyster shell; the low iodine content, during the first 4 weeks, of the eggs from the iodo-salicylic acid group; the more rapid increase in iodine content of the eggs when sodium iodide was fed; and the wide fluctuations in the iodine content of the eggs from the groups receiving desiccated

thyroid, iodo-salicylic acid or sodium iodide. It should also be noted that there was a temporary increase in the iodine content of the eggs laid by the hens given iodo-salicylic acid during the sixth, seventh and eighth weeks. Most of this increase occurred after the amount of iodo-salicylic acid given was doubled at the end of the first 6 weeks. No such increase occurred in the case of the birds given sodium iodide although the amount of iodine given was likewise doubled.

The results obtained in experiment 3 with an equal intake of iodine from iodo-salicylic acid and sodium iodide are similar to those obtained in experiment 2. The iodine content of the eggs from the iodo-salicylic acid group increased more slowly than the iodine content of the eggs from the sodium iodide group and remained lower except during the 11- to 12-week period. In experiment 2, the iodine content of the eggs from the iodo-salicylic acid group was relatively less, except during the seventh and eighth week period, when it was relatively greater (iodine intake considered) than for the sodium iodide group.

In experiment 4 the iodine content of the eggs obtained from any one group was quite consistent but there was a significant difference in the iodine content of the eggs laid by the birds in different groups. Thus the difference between the iodine content of the eggs laid by the birds given the potassium iodide and iodo-salicylic acid was over nine times the standard error of the difference. It is evident that the iodine content of the eggs laid by the birds fed potassium iodide, potassium iodate and di-iodotyrosine was about the same. The eggs from the birds given iodized olive oil and desiccated thyroid were intermediate in iodine content while the iodine content of the eggs from the birds receiving iodo-salicylic acid did not differ significantly from that of the eggs laid by the control group.

In order to determine what variation there was in the amount of iodine contained in the excreta, two analyses were made of composite samples from the different groups in experiment 2 and at 4-week intervals in experiment 3. As shown

in table 2, the percentage of iodine in the excreta of the birds fed the basal ration only and those fed oyster shell was low and did not differ appreciably. A higher percentage of iodine was found in the excreta from the birds given iodo-salicylic acid than from the group fed desiccated thyroid (experiment 2) although both received the same amount of iodine. In experiment 2 the excreta from the birds receiving sodium iodide contained the highest percentage of iodine but the increase over the other groups was not in proportion to the greater amount of iodine given these birds. In experiment 3 the excreta of the birds fed iodo-salicylic acid contained a

TABLE 2  
*Iodine content of the excreta in  $\gamma$  per gram*

IODINE SUPPLEMENT GIVEN	EXPERIMENT 2		EXPERIMENT 3					
	3rd week	6th week	Before	4 weeks	8 weeks	12 weeks	16 weeks	20 weeks
None	3.75	10.1						
Oyster shell	2.01	6.98						
Desiccated thyroid	79.5	22.3						
Iodo-salicylic acid	86.5	131	5.09	4.22	42.6	4.13	5.64	2.3
Sodium iodide	394	253	0.24	3.55	16	0.30	1.73	1.9

higher percentage of iodine than those of the birds given sodium iodide. The results obtained in experiments 2 and 3 thus indicate that the excreta of the birds fed iodo-salicylic acid contained a higher percentage of the total iodine intake than those of birds fed sodium iodide.

Differences in the excretion of iodine were studied further in two balance experiments which were run in order to determine whether there was a difference in the percentage of the total iodine intake recovered in the feces and in the eggs laid. The feed consumed during a period of 1 week was weighed, the feces collected and a composite sample of the feces, and of the eggs laid, analyzed for iodine. The data are summarized in table 3. So far as the iodine content of the eggs is concerned, the results agree with those presented in

table 1. The analyses of the feces also agree with those in table 2 in that the highest percentage of iodine was found in the feces of the birds given iodo-salicylic acid while less was recovered from the feces of the birds fed potassium iodide and least from the feces of the birds fed di-iodotyrosine or 48 % in both trials. If the percentage of iodine in the excreta of the birds given iodo-salicylic acid is compared with that of the birds given potassium iodide or sodium iodide it will be observed that the differences are so consistent that there can be little doubt that more of the iodine fed in the form of iodo-salicylic acid is excreted in the feces.

TABLE 3  
*Summary of iodine balance trails (experiment 4)*

TRIAL	SOURCE OF IODINE	FEED CONSUMED	TOTAL IODINE IN FEED	NUMBER OF EGGS LAID	IODINE PER EGG	TOTAL IODINE IN EGGS	TOTAL IODINE IN FECES	TOTAL IODINE EXCRETED	RECOVERY
		gm.	γ		γ		γ	γ	%
1	Potassium iodide	2120	29,700	18	120	2160	17,200	19,360	66
1	Iodo-salicylic acid	1300	18,200	10	20	200	12900	13,100	71
1	Di-iodotyrosine	2640	37,200	18	125	2250	18000	20,250	55
2	Di-iodotyrosine	2990	42,000	19	125	2400	20,000	22,400	54

#### IODINE IN THE TISSUES

At the end of experiments 2 and 3 representative birds from the groups on the basal and oyster shell ration and all the birds left of the other three groups were killed. Analyses made of the thyroids, ovaries and parts of the thymus of these birds are shown in table 4. It will be observed that the percentage iodine content of the thyroids differs from what might be expected on the basis of the amount of iodine fed, the thyroids of the hens receiving sodium iodide containing only slightly more iodine, on the average, than those of the hens fed the basal ration. There were considerable differences between individuals hence, small differences between the groups cannot be considered significant. The thymus contained an appreciable amount of iodine while the ovaries



(stripped, so far as possible, of all ova) contained very little. The ovaries of the hens given sodium iodide contained significantly more iodine than the others (experiment 2). This was apparently due to the high iodine intake since the iodine con-

TABLE 4

*Iodine content of the thyroid, thymus and ovary in  $\gamma$  per gram of material*

EXPERIMENT NO.	IODINE SUPPLEMENT GIVEN	THYROID			THYMUS		OVARY	
		Number of birds	Iodine content		Number of birds	Average iodine content	Number of birds	Average iodine content
			Average	Range				
2	None	3	1540	1430- 1,710	1	54	1	7
2	Oyster shell	5	1950	830- 4,350	2	1190	1	1
2	Desiccated thyroid	2	3570	1490- 5,650	2	104	2	5
2	Iodo-salicylic acid	4	4920	2610-10,700			2	3
2	Sodium iodide	4	1880	930- 3,590			2	32
3	Iodo-salicylic acid	3	390		1	60	3	1
3	Sodium iodide	4	530		3	49	4	4

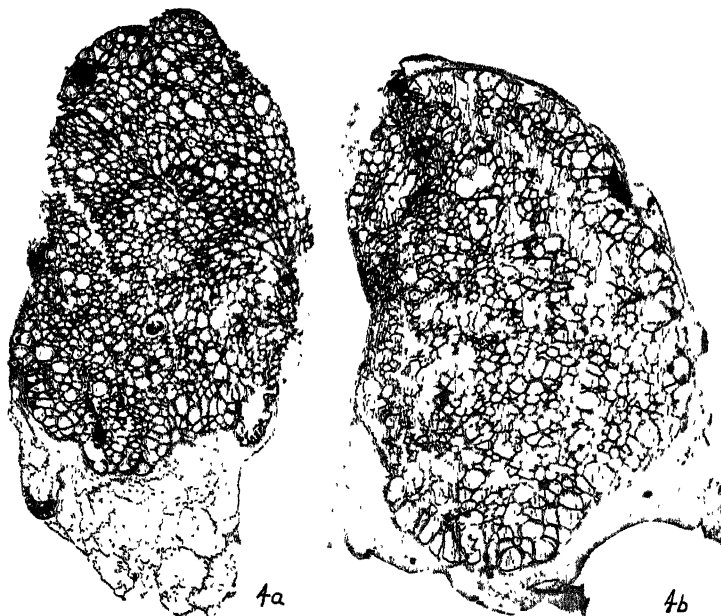


Fig. 4 Thyroid of a bird fed the basal ration only (4a), and of a bird fed the basal ration plus sodium iodide (4b) (experiment 2).  $\times 8$ .

tent of the ovaries from the sodium iodide group in experiment 3 was low.

Histological examination of the thyroids confirms in a general way the analytical results. The lobules in the thyroids of birds fed the basal ration were smaller than the lobules in the thyroids of the birds fed sodium iodide as will be seen by comparing figures 4 a and b.

Table 5 shows the results of iodine analyses of the spleen, pancreas, kidney, fat and blood. The iodine content was low and variable. Thus the relatively high iodine content of the pancreas of the birds given iodo-salicylic acid was due to the

TABLE 5  
*Iodine in  $\gamma$  per gram of material (experiment 3)*

	IODO-SALICYLIC ACID GROUP		SODIUM IODIDE GROUP	
	Number of birds	Average	Number of birds	Average
Spleen	3	1.3	4	2.93
Pancreas	3	3.3	4	0.90
Kidney	3	0.3	4	0.20
Fat	3	1.23	4	0.35
Blood	3	0	4	1.38

high value obtained for one bird only. While the iodine content of the thymus, and other tissues is extremely variable, the data presented show that iodine may occur in appreciable amounts outside the thyroid.

#### DISCUSSION

The results summarized above show that the feeding of moderate amounts of desiccated thyroid decreased feed consumption, body weight and egg production. The loss in body weight observed agrees with the findings reported by others (Hutt, '30; Asmundson, '31). The effects of thyroid feeding observed do not apparently depend upon the total amount of iodine given in the form of desiccated thyroid, which agrees with the conclusion of Moreh ('29) that the activity of the preparation is not closely related to the iodine content. Since

the effects of desiccated thyroid are due to the thyroid hormone it is not to be expected that other iodine preparations would have a similar effect. Nevertheless, when sodium iodide is fed in large amounts the apparent effect is the same as in the case of desiccated thyroid—feed consumption is decreased, after which follows loss of weight and decrease or cessation of egg production.

The amount of iodine in the egg varies according to the form in which it is given to the birds. Thus the iodine content of the eggs laid by birds (experiment 4, table 1) fed sodium iodide, sodium iodate and di-iodotyrosine was significantly higher than that of the eggs from the birds fed iodo-salicylic acid. These results are in agreement with those obtained in experiments 2 and 3 although the results of the earlier experiments were not so clear cut. Considering all the data there can be no doubt that, contrary to the results reported by Wilder, Bethke and Record ('33), the form in which the iodine is given does influence the amount of iodine in the eggs laid. It follows from this that if the iodine in the various rations is from different sources, the iodine content of the eggs will not necessarily vary according to the amount of iodine fed. When, however, the same form of iodine is fed at different levels it might be expected that the amount of iodine in the eggs would vary with the amount of iodine in the feed. Contrary to this the data in table 1 for birds fed sodium iodide at different levels (experiments 2 and 3) indicate that the iodine in the egg does not vary in direct proportion to the iodine intake. Our results, while not conclusive on this point, agree with those reported by Almquist and Givens ('35) and disagree with those of Wilder, Bethke and Record ('33).

The data in tables 1, 2 and 3 indicate that if the iodine content of the excreta is high, or the percentage of the total iodine intake recovered in the feces is high, the iodine content of the eggs is relatively low. The percentage of iodine recovered from the feces was higher when iodo-salicylic acid was fed than when potassium iodide or di-iodotyrosine was

fed. The results of the various analyses also point to the conclusion that relatively more of the iodine is excreted when it is fed in the form of iodo-salicylic acid than if sodium or potassium iodide are fed.

The percentage of iodine in the thyroid may also vary with the source since there was relatively less storage of iodine in the thyroids of the birds fed sodium iodide than in the case of the birds given iodo-salicylic acid or desiccated thyroid. The percentage of iodine in the thyroids of the birds fed oyster shell, desiccated thyroid and sodium iodide was about the same as reported by Cruickshank ('29) for laying hens while the values for the basal group were lower and those for the birds receiving iodo-salicylic acid were higher. When the iodine intake from iodo-salicylic acid and sodium iodide was low (experiment 3) the results were somewhat different. Evidently, many factors influence the iodine content of the thyroid, hence no definite conclusions can be drawn with respect to the effect of feeding different forms of iodine on the amount of iodine in the tissues, until more extensive data are available.

#### SUMMARY

White Leghorn hens were supplied with iodine from oyster shell, desiccated thyroid, sodium iodide, potassium iodide, iodo-salicylic acid, di-iodotyrosine and iodized olive oil. The birds were weighed at regular intervals and iodine determinations were made on samples of eggs from all the groups and on the feces and the thyroids and other tissues from some of the groups.

The body weight, egg production and feed consumption was not adversely affected except in the case of the birds fed desiccated thyroid and in the case of the birds given sodium iodide when they received 16,000  $\gamma$  of iodine per bird daily.

The iodine content of the eggs varied from 3 to 1730  $\gamma$  per egg. It differed according to the source of the iodine but corresponded only approximately to the iodine intake. Thus the iodine content of the eggs laid by birds fed potassium

iodide, potassium iodate and di-iodotyrosine was significantly higher than the iodine content of the eggs laid by the birds fed iodo-salicylic acid. When iodo-salicylic acid was fed a higher percentage of the total iodine ingested was excreted than when di-iodotyrosine and potassium (or sodium) iodide was fed. These results indicate that iodine utilization by laying hens, in so far as it can be measured by the iodine content of the eggs and the excreta, depends upon the source as well as the amount of iodine given.

The iodine content of the thyroid varied considerably but the differences were not statistically significant. The thymus also contained appreciable amounts with less iodine in the ovary (stripped of yolks), spleen and other tissues.

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# VITAMIN C STUDIES WITH CHILDREN OF PRESCHOOL AGE

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ONE FIGURE

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The recently developed chemical methods for determining with considerable accuracy the amount of vitamin C (ascorbic acid) in biological material have made possible studies of vitamin C retentions in children. Hitherto, in the few studies pertaining to the ascorbic acid needs of children, comparisons have been made between the amount ingested, as orange juice, and the urinary output. In some cases, this has been estimated from urine samplings; in others, from the 24-hour excretions. Without knowledge of the ascorbic acid content of all the foods eaten, it is obvious that actual needs may be higher than the figure thus obtained. Furthermore, the method of preserving the urines in some of the studies suggests that deterioration of the ascorbic acid may have occurred, thus accounting for certain of the low values.

The purpose of the present study was to determine how much vitamin C is needed by children of the preschool age. The investigation was made with three normal boys aged 39, 57 and 59 months, respectively, at the beginning of the study. During the investigation the children lived under controlled conditions, as described in a previous report (Daniels et al., '34). Each study period consisted of 15 days, a 5-day preliminary period for physiological adjustment, and two 5-day balance periods. During the preliminary period also, retentions were determined but since the urinary ascorbic acid

output was apt to be irregular, depending, it would seem, upon the degree of saturation of the tissue of a given child, these balances have not been included.

The food served was prepared in three lots, one for the preliminary and one for each of the two balance periods. Cooked foods were stored in closely covered porcelain containers in a low temperature refrigerator ( $8^{\circ}\text{C}.$ ), a weighed portion being removed each day for serving. The orange juice, which contained considerable pulp, was prepared at the beginning of each period by extracting both juice and pulp with a glass hand press and subsequently straining through a wire (16 mesh) sieve. This was immediately stored in air-tight jars in the low temperature refrigerator.

The ascorbic acid content of the foods was determined by means of the 2:6 dichlorophenolindophenol method described by Birch, Harris and Ray ('33), with modifications suggested by Tauber and Kleiner ('35) and Ahmad ('35), 10 gm. samples of the more solid foods and 30 gm. samples of the daily aliquot milk mixtures being used in each case. The foods were thoroughly ground with purified sea sand, and 10 to 15 cc. of 10 % trichloroacetic acid, the extracted material being decanted into a large centrifuge tube. Second and third extractions were similarly made. In all, 45 cc. of the trichloroacetic acid were used for each sample of material. The combined extractions were centrifuged at high speed, the clear solution was decanted, made to a definite volume and titrated immediately against a standard solution of 2:6 dichlorophenolindophenol, the average of six titrations being taken as the final value. The ascorbic acid content of orange juice diluted 1:10 was similarly determined. Tests of the cooked foods at the beginning and end of the metabolism period, and of the orange juice each day indicated that under the conditions of storage no deterioration of vitamin C had occurred.

Throughout the period of study, which was continuous from September 29th to February 15th, with the exception of 4 days between each controlled period, and the Christmas holidays, the kind of food, method of preparation, and with few exceptions, the amounts served to each child were the same during

each of the several periods. Foods were eaten quantitatively. Twice only was it found necessary to increase the caloric ingestions, each time increases being made with those foods (apple sauce, potatoes and stick candy) which contained little or no vitamin C. The daily orange juice was divided into two portions and given between meals. To this were added 3.7 cc. of a standard cod liver oil and 4 drops of viosterol. A typical

TABLE 1  
*Food and vitamin C intake of children, January 14th to 20th*

	G.E.		F.V.		J.E.	
	Amount	Ascorbic acid	Amount	Ascorbic acid	Amount	Ascorbic acid
	gm.	mg.	gm.	mg.	gm.	mg.
Orange juice	60.0	43.6	60.0	43.6	60.0	43.6
Banana	61.0	11.9	76.0	14.9	61.0	11.9
Milk	732.0	5.9	732.0	5.9	732.0 <sup>1</sup>	5.9
Green beans	70.0 <sup>1</sup>	2.4	70.0 <sup>1</sup>	2.4	90.0 <sup>1</sup>	3.1
Potato	80.0 <sup>1</sup>	2.5	80.0 <sup>1</sup>	2.5	80.0 <sup>1</sup>	2.5
Carrots	56.0 <sup>1</sup>	1.8	56.0 <sup>1</sup>	1.8	75.0 <sup>1</sup>	2.4
Prunes	20.0 <sup>1</sup>	0	53.3 <sup>1</sup>	0	53.3 <sup>1</sup>	0
Cereal, dry weight	13.8	0	13.8	0	13.8	0
Bread	49.0	0	51.0	0	51.0	0
Butter	15.0	0	18.0	0	17.0	0
Egg	80.0	0	110.0	0	110.0	0
Sugar	21.0	0	21.0	0	19.0	0
Beef	40.0	0	60.0	0	70.0	0
Apple sauce	70.0 <sup>1</sup>	0	91.0 <sup>1</sup>	0	70.0 <sup>1</sup>	0
Cod liver oil	7.5	0	7.5	0	7.5	0

<sup>1</sup> Weight and ascorbic acid content after cooking.

day's food intake for one period, January 14th to 20th, with the ascorbic acid value of each food served, is given in table 1. Variations in the amounts of vitamin C were obtained by increasing the orange juice, and by adding to the orange juice just previous to serving a solution of commercial ascorbic acid<sup>1</sup> as indicated.

Urines were collected quantitatively, each voiding being preserved with 2 cc. of toluene and from 2 to 4 drops of concentrated sulphuric acid, depending upon the amount of

<sup>1</sup> Redoxon, obtained from Hoffmann-La Roche, Inc., Nutley, N. J.



orange juice taken, thus keeping the pH between 2.7 and 3. Urine specimens were placed immediately on ice, in a closed chest, and subsequently (twice each day) were transferred to a low temperature refrigerator (5°C.). At the end of each 24 hours, the urines were pooled and the ascorbic acid content, by means of the 2:6 dichlorophenolindophenol method was determined with unfiltered urines, since it had been shown that filtering under the conditions tested resulted in some destruction of the ascorbic acid contained therein. Tests of urines to which known amounts of commercial ascorbic acid were added gave recoveries of 97 to 102 %.

That the methods used for preserving and storing the urines were satisfactory and allowed for no appreciable destruction of the ascorbic acid during the collection periods is indicated by comparisons of tests made after the urines had been stored 24, 48 and 72 hours, respectively. Tests with the same urine similarly stored, but preserved with glacial acetic acid, confirmed the findings of Johnson and Zilva ('34) to the effect that some destruction occurs within 24 hours; this increases with longer storage. Urines preserved with sulfuric acid in quantities to bring the pH to 2.7 to 3 were found to be stable at least 90 hours, the longest testing period.

Comparative tests of the ascorbic acid content of urines preserved as indicated with sulfuric acid by the two methods recommended: 1) titration against 2:6 dichlorophenolindophenol (Birch, Harris and Ray, '33); and 2) colorimetric determinations with phospho-18-tungstic acid (Medes, '35), which rules out possible phenols and thiol compounds were found to be in close agreement, the range of difference ( $-6.5$  to  $+9.6$  %) being within the limits of error of the methods.

## RESULTS

The ascorbic acid retentions of the children studied have been grouped in order of ingestions per kilogram of body weight, including only those values obtained subsequent to the adjustment periods. Since, in some instances, a fair proportion of the ascorbic acid intake was obtained from foods other

than orange juice and commercial ascorbic acid when used, the amount of the vitamin from the several sources is listed separately. When only 1 ounce of orange juice ( $22 \pm$  mg. of ascorbic acid) was given, slightly more than one-half of the total vitamin C intake was obtained from the other foods served; when 4 ounces of orange juice ( $90 \pm$  mg. of ascorbic acid) were taken, from one-fourth to one-fifth was furnished from other sources.

The ascorbic acid ingestions ranged from 2.7 to 12.7 mg. per kilogram of body weight. Following the 5-day preliminary period, the urinary excretions, which were significantly constant from day to day, tended to parallel the ingestions. In no case were the very low excretions reported by other workers (Hess and Benjamin, '34; Harris and Ray, '35) found, 10.6 mg. being the lowest obtained in any metabolism period. This followed an ingestion of 3.1 mg. per kilogram with the youngest child studied (G.E., 12/15).

Retentions based on the assumption that all excess of intake is excreted through the kidneys, ranged between 1.7 and 4.3 mg. per kilogram, and in general, paralleled the ingestion up to 7.5 mg., or thereabout per kilogram. With the highest ingestions tested, 10.7 to 12.7 mg. per kilogram, no more vitamin C was retained than with the next lower level tested, namely, 7.5 mg. per kilogram, suggesting that this latter amount is the minimum for children of the ages studied. Depleted tissues will retain more until they become saturated. This is shown by the daily urinary excretion of the three children immediately following the 3-week holiday period at home (table 3). With a total ingestion of 68.1 mg., G.E. excreted an average of 11.1 mg. per day during 5 successive days of the preliminary period, whereas during the following 6-day period, he excreted an average of 20.4 mg. F.V. and J.E. also excreted somewhat less during these preliminary days, suggesting that in all three cases the home diets of the children contained less than the optimum amount of vitamin C. On the other hand, when a diet fairly high in vitamin C followed one which had been shown to be adequate (table 4) the day-by-day urinary excretion remained constant during the period of study.

TABLE 2  
The relation of ascorbic acid ingestion to ascorbic acid retention

NAME	DATE	WEIGHT kg.	CREATININE mg.	INTAKE				URINARY EXCRETION			RETENTION			
				Orange juice	Food	Redoxon	Total	Per kilo-gram	Per milligram creatinine	Average	Range	Total	Per kilo-gram	Per milligram creatinine
J.E.	12/15	19.0	339	22.5	29.4	mg.	51.9	mg.	0.15	14.6	13.0-15.8	37.3	mg.	0.11
F.V.	12/15	19.3	293	22.5	30.7	..	53.2	2.8	0.18	15.6	13.3-17.6	37.6	2.0	0.13
G.E.	12/15	16.3	245	22.5	28.0	..	50.5	3.1	0.21	11.6	10.6-13.3	38.9	2.4	0.16
J.E.	1/19	19.2	346	38.9	25.0	..	63.9	3.3	0.19	25.0	19.0-37.5	38.9	2.0	0.11
F.V.	1/19	18.6	294	38.9	25.1	..	64.0	3.4	0.22	17.2	15.4-20.3	46.8	2.5	0.16
J.E.	1/14	19.2	365	43.6	25.8	..	69.4	3.6	0.19	36.1	24.2-52.8 <sup>1</sup>	33.3	1.7 <sup>1</sup>	0.09 <sup>1</sup>
J.E.	9/29	18.0	318	48.0	18.8	..	66.8	3.7	0.21	20.3	13.8-26.8	46.5	2.6	0.15
J.E.	11/26	18.9	348	48.0	23.7	..	71.7	3.8	0.21	29.7	26.7-35.3	42.0	2.2	0.12
G.E.	1/19	16.2	254	38.9	22.6	..	61.5	3.8	0.24	17.8	17.1-18.9	43.7	2.7	0.17
F.V.	11/26	18.7	297	48.0	25.1	..	73.1	3.9	0.25	26.5	24.0-28.2	46.6	2.5	0.16
F.V.	1/14	18.4	287	43.6	27.4	..	71.0	3.9	0.25	16.7	13.3-19.7	54.3	3.0	0.19
J.E.	12/1	19.0	343	48.0	27.2	..	75.2	4.0	0.22	29.5	27.8-30.0	45.7	2.4	0.13
F.V.	12/1	18.7	296	48.0	28.2	..	76.2	4.1	0.26	25.8	25.0-27.3	50.4	2.7	0.17
F.V.	9/29	16.8	272	48.0	20.5	..	68.5	4.1	0.25	23.0	21.0-24.0	45.5	2.7	0.17
G.E.	1/14	16.2	249	43.6	24.5	..	68.1	4.2	0.27	20.3	15.2-25.5	47.9	2.9	0.19
G.E.	9/29	14.9	232	48.0	18.8	..	66.8	4.5	0.29	23.2	13.6-29.3	43.6	2.9	0.19
G.E.	11/26	15.8	249	48.0	22.9	..	70.9	4.5	0.28	21.5	19.7-24.0	49.4	3.1	0.20
G.E.	12/1	16.0	251	48.0	25.7	..	73.7	4.6	0.29	22.8	21.5-25.8	50.9	3.2	0.20
J.E.	10/20	18.3	323	45.0	18.1	48	111.1	6.1	0.34	45.9	41.4-50.0	65.2	3.5	0.20
J.E.	10/15	18.3	347	47.2	19.1	48	114.3	6.2	0.33	57.8	50.0-66.7	56.5	3.1	0.16
J.E.	11/10	18.6	350	90.0	30.7	..	120.7	6.4	0.34	56.2	52.2-61.5	64.5	3.5	0.18
J.E.	11/5	18.6	350	96.0	22.7	..	118.7	6.4	0.34	51.3	51.1-53.3	67.4	3.6	0.19
F.V.	10/20	17.5	286	45.0	20.2	48	113.2	6.5	0.40	58.4	51.1-66.7	54.8	3.1	0.19
F.V.	11/5	18.1	295	96.0	24.2	..	120.2	6.6	0.41	51.6	51.1-52.2	68.6	3.8	0.23
F.V.	10/15	17.3	286	47.2	21.2	48	116.4	6.7	0.41	66.6	61.3-75.0	49.8	2.9	0.17
F.V.	11/10	18.2	293	90.0	32.8	..	122.8	6.7	0.42	56.0	54.5-57.1	66.8	3.7	0.23
G.E.	10/20	15.2	239	45.0	17.2	48	110.2	7.3	0.46	56.0	52.2-60.0	54.2	3.6	0.23
G.E.	10/15	15.1	240	47.2	18.7	48	113.9	7.5	0.47	56.4	50.0-61.5	57.5	3.8	0.24
G.E.	11/5	15.5	242	96.0	20.7	..	116.7	7.5	0.48	51.2	48.0-54.5	65.5	4.2	0.27
G.E.	11/10	15.6	243	90.0	27.7	..	117.7	7.5	0.48	54.3	51.1-57.1	63.4	4.1	0.26
J.E.	2/9	19.4	335	87.3	24.3	96	207.6	10.7	0.62	142.4	140.8-145.4	65.2	3.4	0.19
F.V.	2/9	19.1	293	87.3	25.0	96	208.3	10.9	0.71	149.5	144.0-156.0	58.8	3.1	0.20
J.E.	2/4	19.3	333	90.0	26.5	96	212.5	11.0	0.64	148.1	137.1-160.0	64.4	3.3	0.19
F.V.	2/4	19.0	288	90.0	27.8	96	213.8	11.3	0.74	151.2	143.4-157.9	62.6	3.3	0.22
G.E.	2/9	16.7	255	87.3	23.2	96	206.5	12.4	0.81	134.2	128.0-143.2	72.3	4.3	0.28
G.E.	2/4	16.6	255	90.0	25.4	96	211.4	12.7	0.83	147.6	141.2-160.0	63.8	3.8	0.25

<sup>1</sup> Child had slight cold with elevated temperature 1 day. Increased excretion of creatinine during 1 day, and vitamin C during 2 days. Two and five-tenths grams of aspirin given two successive nights.

The highest retentions, 4.1, 4.2 and 4.3 mg. per kilogram, following ingestions of 7.5, 7.5 and 12.4 mg. per kilogram, respectively, were obtained with the youngest child, G.E. (39 months of age at the beginning of the investigation). That these high retentions were not found with the other children,

TABLE 3

*Daily intake and excretion of vitamin C following a 3-week period of home diet*

DATE	G.E.	F.V.	J.E.
	Intake: 68.1 mg.	Intake: 71.0 mg.	Intake: 69.4 mg.
	Urinary vitamin C	Urinary vitamin C	Urinary vitamin C
	mg.	mg.	mg.
January 8	11.3	12.2	18.9
9	11.1	10.6	19.5
10	9.4	14.5	18.1
11	10.4	14.6	23.8
12	13.2	19.0	27.5
13	23.4	15.2	27.0
14	23.5	13.3	25.5
15	21.0	17.1	24.2
16	20.0	17.1	....
17	15.2	16.4	....
18	19.5	19.7	....

TABLE 4

*Urinary excretion of vitamin C during a high intake and a subsequent lower intake*

F.V.			
Intake: 122.79		Intake: 73.09	
Date	Urinary vitamin C	Date	Urinary vitamin C
	mg.		mg.
11/10	57.14	11/20	28.23
11/11	55.38	11/21	28.08
11/12	54.54	11/22	28.62
11/13	55.81	11/23	23.29
11/14	57.14	11/24	27.45

although seemingly a plethora of vitamin C was given during certain periods, suggests that young tissues may have greater need for vitamin C than more mature tissues. Hess and Benjamin found that infants excreted very little vitamin C even though they were receiving diets which would seem to contain adequate amounts. "Only when excessively large doses

of vitamin C were fed (1 pt. orange juice daily) did appreciable urinary excretion occur." In seeming corroboration of the possible greater demand of young tissues are the findings of Bessey and King ('33) to the effect that in younger animals the vitamin C content of the tissues was higher than that of older animals. The human tissues tested also were found to contain more vitamin C than those of the adult (Bessey and King, '33; Yavorsky, Almaden and King, '34).

Estimating vitamin C retentions on the basis of creatinine eliminations of the children studied, further suggests that the younger child retains more than the older (table 2). G.E., with a creatinine elimination of 255 mg., retained 0.25 and 0.28 mg. of ascorbic acid per milligram of creatinine during two successive periods (2/4 and 2/9, respectively) when receiving the highest ingestions, whereas F.V., with creatinine eliminations of 288 and 293 mg. retained 0.22 mg. and 0.20 mg. per milligram of creatinine, respectively, while J.E. (2/4 and 2/9) with the highest creatinine elimination, retained only 0.19 mg. of vitamin C per milligram of creatinine, when receiving what would seem to be considerably more than sufficient to cover requirements. This greater demand of the younger tissue for vitamin C may be concerned with the more rapid oxidation processes of this age. The fact that during anesthesia there is an increased output (Zilva, '35; Bowman and Muntwyler, '35) also points to a relationship between oxidation needs and vitamin C. More work is needed to confirm these findings.

That commercial ascorbic acid can be used for supplying the vitamin C need of human tissues has been indicated by a number of investigators (Svensgaard, '34; Goettsch, '35; Hawley, Stephens and Anderson, '36). Further confirmation of this is shown in the balance studies with the children, when synthetic ascorbic acid was used as part of the vitamin requirement. The total ingestions of this period (10/15) were comparable to those of the subsequent period (11/5) in which the vitamin was supplied wholly from food. Urinary excretions and retentions of ascorbic acid were very similar during

both periods; whereas retentions were considerably lower, between 1.7 and 3.1 mg. per kilogram, respectively, during the periods (9/29, 11/26 and 1/14) in which similar amounts of vitamin C were furnished from foods, but none from the synthetic ascorbic acid. The higher retentions of the former periods would seem to be due to the increase in the ascorbic acid, part of which was furnished by the commercial preparation.

#### COMMENTS

The similarity of response of the three children receiving comparable amounts of ascorbic acid at various levels of ingestion would seem to attest to the validity of the findings

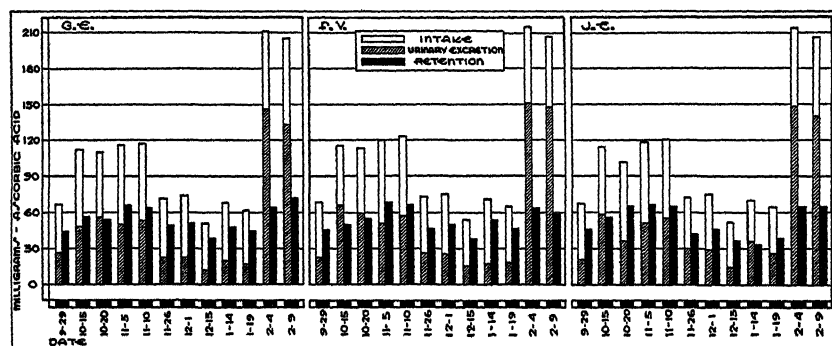


Fig. 1 The relation of intake to urinary excretion and retention of vitamin C in children.

(fig. 1). With the exception of the very highest levels tested, retentions were directly related to ingestion, indicating some rather definite relationship between vitamin C and tissue needs. At all levels of ingestion, urinary excretions tended to parallel ingestions. The explanation for this is not clear. When less than optimum amounts are taken for any considerable period, it would seem that tissue demands would reduce excretion to a minimum, whereas even at the lowest levels of ingestion tested, ascorbic acid was found in appreciable amounts. Tests for interfering thiol compounds (Shinohara and Padis, '36) indicated that at most only approximately 3 mg. of ascorbic acid equivalent could be thus

accounted for—too little to explain the difference in the urinary excretions. During each period of study, the children received the same foods in the same amounts with the exception of orange juice and the commercial ascorbic acid. Thus the urinary excretion of any interfering substances should be the same during all periods for a given child. Nor does it seem probable that the method of giving the ascorbic acid so flooded the system that less would be retained during some periods than others. The orange juice was always given under the same conditions, therefore storage with all levels of ingestion would be equally possible.

Urinary excretion is believed to represent excess over needs. What then is the explanation for excretions with the low retentions tested? Under normal conditions, the demand for tissue vitamin apparently is constant in a given individual. Scurvy develops when the stores of vitamin C are exhausted, and therefore the urinary vitamin C becomes an index of the degree of saturation of the tissue. "Further studies may reveal hitherto unrecognized pitfalls in the methods used in determining the vitamin C content of the urine" (Hawley, Stephens and Anderson). On the otherhand, ascorbic acid metabolism may be comparable to nitrogen metabolism: Urinary excretion representing both endogenous and exogenous acid. Until the tissues become completely exhausted, the endogenous vitamin C will always appear in the urine, high urinary outputs with less than optimum ingestions being due to the fact that the organism adjusts slowly to different levels of ingestion.

#### SUMMARY

In an attempt to determine the vitamin C needs of young children, three boys of preschool age were given during twelve successive periods constant weighed diets differing from period to period only in the amount of ascorbic acid contained therein. A 5-day preliminary period for physiological adjustment preceded each two consecutive 5-day metabolism periods. During four periods, a part of the vitamin was supplied by a synthetic commercial preparation.

1. Under the conditions of the investigation, urinary excretion of ascorbic acid paralleled the intake, notwithstanding the fact that retentions at certain levels of ingestion were considerably below the physiological optimum.

2. Retentions of ascorbic acid paralleled the ingestions only up to 7.5 mg. or thereabout per kilogram. Higher ingestions (10 to 12 mg. per kilogram) were without influence on the retentions of the children studied.

3. The highest retentions estimated either on the basis of weight (3.8 to 4.3 mg. per kilogram), or creatinine elimination (0.24 to 0.28 mg. per milligram of creatinine), were obtained with the youngest child, suggesting that there is a greater demand by younger tissue for more vitamin C.

4. Comparable amounts of commercial ascorbic acid and ascorbic acid from foods resulted in similar retentions.

5. The relation of ascorbic acid intake to excretion and retention is discussed.

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# STUDIES ON THE RELATION OF DIET TO GOITER

## IV. THE ANTIGOITROGENIC VALUE OF SOME FOODS

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### ONE FIGURE

According to evidence accumulated over more than 80 years, enlarged thyroid glands are most frequent in those populations whose environment is relatively deficient in iodine. Goitrous areas have been reported to exist in practically every country. Attempts have been made to arrive at some value for the daily human iodine requirement by analysis of food and water from goitrous and non-goitrous regions, or from the daily iodine excretion of persons living in such regions. Results are conflicting, but a probable value is 50 to 100  $\gamma$  per day. Lunde ('29) found daily elimination of 40  $\gamma$  at Hosstvedt, Norway (60 % goiter in children), one of 61  $\gamma$  at Ruud (30 % goitrous) and 173  $\gamma$  at Viki Sojn, where goiter is absent. He believes that the daily human requirement is at least 50  $\gamma$ , possibly as high as 100  $\gamma$ . Oppenheimer ('29) concluded from the work of Fellenberg and others that the average human requirement from all sources is 100  $\gamma$  per day. Mazzocco ('29) calculated that the average daily intake of inhabitants of Salta, Argentina, a goitrous area, is less than 40  $\gamma$ . In one of the previous papers of this series (Levine, Remington and von Kolnitz, '33 b), it was shown that goiter could be effectively prevented in young rats by supplying 1 to 2  $\gamma$

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of iodine per day as iodide, and that this corresponded, on a basis of caloric intake, to 60 to 120  $\gamma$  for the human. There is quite an extensive literature on the iodine content of food, water, and soil from all parts of the world.

Until quite recently it has been assumed that, with respect to trace elements (e.g., iron, copper, manganese, iodine, etc.), the nutritional value of foods was indicated by chemical analysis. Some have even gone farther than this, and argued that these elements as found in food of plant or animal origin, should be more readily and completely utilized in the diet, than inorganic forms. That neither of these generalizations is wholly valid is shown, with respect to iron, by Elvehjem ('32), who reported that the iron of hematin is less completely utilized than that of ferric chloride, and by Sherman, Elvehjem and Hart ('34 b), who found the iron of spinach, oysters, alfalfa, and blood only 25 % available. The amount available for hemoglobin building in the rat paralleled very closely the amount present in inorganic form as determined by the dipyrindyl method. These same authors ('34 a) found the copper of egg yolk inferior to copper sulfate. The calcium of spinach is very poorly utilized as compared with that of milk, according to a recent report by Fincke and Sherman ('35). With regard to iodine, the physiological requirement for which is much lower than that of any other known inorganic factor, few and inconclusive experiments have been made. Fellenberg ('23), using himself as a subject, and a very low iodine intake, reported that retention in the body is in the following descending order: Iodized fats, iodides, iodine in vegetables, iodine in animal foods. Pfeiffer ('29) raised radishes on an iodine-enriched soil, and fed the leaves, parallel to potassium iodide, to dogs. He found greater retention in the body from iodide, but of the iodine retained, much more was stored in the thyroid when radish leaves were fed.

In the process of developing our technique for the study of low iodine goiter in the rat (Remington and Levine, '36) to a point where it is possible to evaluate the response to different forms or compounds of iodine, we have fed different

iodine-bearing foods for the purpose of estimating their efficiency in goiter prevention. In this paper are reported preliminary results on some sea foods, and on dried milk. Coulson ('35) has recently redetermined the iodine content of a number of edible sea fish and crustaceans. Among these, haddock was found to be extremely high in iodine, showing an average value, fresh basis, of 5130 parts per billion in the muscle. Coulson ('34) has also made iodine analyses on sixty-four samples of Atlantic and Gulf Coast oysters, the average of which was 492 parts per billion, fresh basis. Samples of these products, which had been dried, ground and analyzed, were on hand in the laboratory. We also had available samples of dried milk from cows in a non-goitrous region,

TABLE 1  
*Iodine content of supplements fed (parts per billion)*

High iodine milk no. 1020	900
High iodine milk no. 1221	1170
Oysters	3200
Haddock	32600
Irish moss	350000
Low iodine milk	ca. 190-260

and prepared by roller process in a semi-commercial plant (Remington and Supplee, '34), and in which analysis had shown a relatively high iodine content.<sup>3</sup> Samples of these products were therefore used in the experiments herein reported, their iodine content being shown in table 1.

#### EXPERIMENTAL

Four series of feeding experiments were run on different dates, using our goitrogenic diet GP (Levine, Remington and von Kolnitz, '33 a) as a basal diet, details and results being shown in table 2. In series I and II, the products to be tested were incorporated in the diet at the levels shown, the completed diets analyzed and the average daily iodine intake calculated from the food consumption. In series III the

<sup>3</sup>Special Dryco manufactured by the Dry Milk Company at Newberry, S. C.

TABLE 2  
*The goiter-preventing value of certain iodine-containing foods. Experimental period 35 days*

	SUPPLEMENT FED	IODINE INTAKE <i>γ per day</i>	NUMBER OF RATS	INITIAL AND FINAL WEIGHT <i>gm.</i>	FRESH THYROID WEIGHT <i>mg. per 100 gm. body weight</i>	DRY MATTER IN THYROID	IODINE IN THYROID DRY BASIS
						<i>%</i>	<i>%</i>
Series I	None	0.13	9	61-114	23.9 ± 0.88	22.7	0.015
	2.8 % low iodine milk	0.18	9	59-133	19.8 ± 0.59	24.4	0.021
	0.86 % oysters	0.35	9	62-122	16.7 ± 0.53	25.2	0.038
	20.43 % low iodine milk	0.44	9	59-169	15.5 ± 0.58	25.9	0.053
	2.80 % high iodine milk	0.37	9	60-141	15.5 ± 0.52	26.1	0.059
	6.45 % oysters	2.09	9	61-152	13.2 ± 0.26	27.0	0.114
	20.43 % high iodine milk	2.27	9	59-176	11.0 ± 0.37	28.7	0.113
Series II	Potassium iodide	3.06	9	60-115	12.1 ± 0.34	31.3	0.200
	None	0.13	8	63-133	24.2 ± 1.30	22.6	0.010
	Potassium iodide	3.20	6	60-111	14.3 ± 0.52	29.9	0.285
	5.50 % oysters	1.98	8	56-137	14.1 ± 0.54	25.6	0.135
	15.00 % high iodine milk	2.14	8	64-172	13.6 ± 0.40	27.3	0.138
Series III <sup>1</sup>	15.00 % low iodine milk	0.53	8	58-161	18.5 ± 0.70	23.7	0.065
	None	0.15	15	58-123	29.2 ± 1.34	21.5	0.022
	0.16 gm. oysters	0.65	8	60-153	16.8 ± 0.81	25.5	0.041
	0.32 gm. oysters	1.15	8	64-170	14.9 ± 0.84	26.1	0.062
Series IV <sup>1</sup>	0.64 gm. oysters	2.15	8	60-163	14.8 ± 0.43	27.1	....
	None	0.15	9	56-129	24.4 ± 0.67	20.0	0.034
	0.38 % haddock	1.45	9	55-127	10.1 ± 0.28	32.8	0.113
	0.76 % haddock	2.70	9	55-126	9.6 ± 0.59	31.5	0.142
	0.036 % Irish moss	1.46	9	55-123	16.4 ± 1.20	26.2	0.066
	0.072 % Irish moss	2.65	9	53-116	14.1 ± 0.45	28.7	0.086
	Potassium iodide	2.80	8	57-119	10.7 ± 0.42	32.6	....

<sup>1</sup> In series III and IV the basal diet was not analyzed, but 0.15 γ per day was added to the iodine yielded by the supplement.

oysters were fed separately, daily, in weighed doses. In series IV the dried haddock and Irish moss were added to the basal diet in the proportions shown in the table, and the iodine calculated on the assumption that the basal diet contained 15 parts per billion. Since the amounts of dried milk necessary to yield a preventive dose of iodine were rather high, we also fed samples of commercial milk powder from a more goitrous region, and which was relatively low in iodine.<sup>4</sup> This low iodine milk was not analyzed, but its iodine content was calculated from that of the mixed diets containing it.

In the third paper of this series (Remington and Levine, '36) it has been shown that in low iodine goiter in the rat, certain relationships exist between the iodine content of the thyroid and its fresh weight, and also between dry matter content and weight, have drawn curves showing these relationships, and have proposed that in any given experiment, we are dealing with an iodine deficient condition if the points fall on these two curves. For the purpose of demonstrating how this may be done, and substantiating our argument as to the goiter-preventing potency of these foods, we have reproduced in figure 1 the curves from the previous paper, on which we have placed the points for the values obtained in the present series of experiments. As can be seen, the agreement is excellent, and establishes not only that the enlarged glands obtained are due to iodine deficiency, but also that the reduction in size obtained by feeding iodine-bearing foods is parallel to the storage of iodine, and decrease of edema, in the glands.

For the purpose of evaluating the relative antigoitrogenic properties of foods as compared with one another, or with iodides, it is necessary to show some relationship between amount of iodine fed, and either iodine content of gland or size of gland. In the earlier stages of our work it was hoped that we could establish permanent reference curves for these relations, which would be valid in all subsequent experiments where the same basal diet was used. As previously pointed

<sup>4</sup> Klim, manufactured by The Borden Company, at Perry, N. Y.

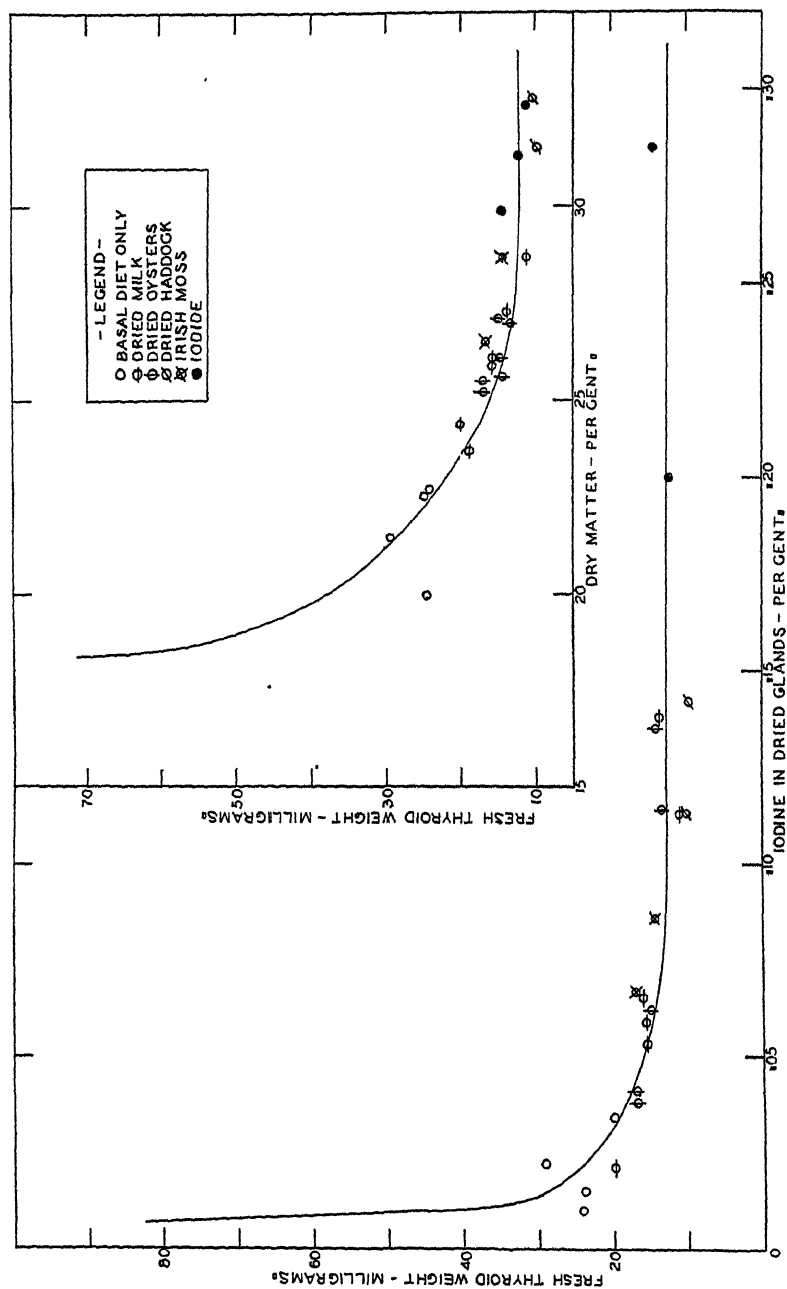


Fig. 1 Showing correlations between thyroid size and iodine content, and thyroid size and dry matter content, in rats fed iodine-bearing foods. Thyroid weight expressed in milligrams per 100 gm. body weight.

out, however, the iodine requirement of the rat is so low, and unknown disturbing factors so difficult to guard against, that the only rational basis of comparison seems to be to place a group of animals on the unsupplemented basal diet in parallel with each experiment, using the same batch of diet, and keeping all other factors as nearly constant as possible.

The experiments reported here were begun before we had arrived at this conclusion, and hence simultaneous iodide feeding within the critical range was not done. The evaluation of these foods in terms of iodide can therefore be deduced only in a general way by comparison with our previously reported experiments on iodine requirement. In that work, rats on the unsupplemented diet produced glands averaging 50 mg. per 100 gm. body weight, whereas in the present work they weighed only 24 to 29 mg., the difference, we believe, being due to the animals securing approximately 0.1  $\gamma$  per day of iodine from an unknown source. Rats of the earlier series, receiving 0.48  $\gamma$  of iodine per day from basal diet and iodide, yielded glands weighing 18.4 mg. per 100 gm. body weight, of 25.1 % dry matter and 0.049 % iodine. Comparing these values with those obtained for milk and oysters in series I and II where the *known* iodine intake was 0.35 to 0.44  $\gamma$  per day, we find that the glands are somewhat smaller (15.5 to 16.7 mg.), the iodine content (0.038 to 0.059 %) and the dry matter content (25.2 to 26.1 %) of about the same order of magnitude. The results indicate that the thyroid response to iodine as it occurs in oysters and milk is of the same order of magnitude (within about 20 %) as that to iodide, and that the claimed superiority in the utilization of 'food iodine,' when fed at minimal effective doses, is not supported by these experiments.

We can also compare the different foods used in a given experiment with one another, and can draw conclusions as to whether or not a given food possesses goiter-preventing properties which may be correlated with its iodine content. For instance, in series I and II, which were done at approximately the same time, and in which the negative controls showed the same degree of goiter, we can compare oysters,



high iodine milk, and low iodine milk, at a daily intake level of approximately 0.4  $\gamma$  of iodine, and again at about 2.0  $\gamma$ . Such a comparison is shown in table 3, an inspection of which leads to the following conclusions:

1. The goiter preventing properties of oysters and milk are in proportion to their iodine content.

2. No significant differences in the availability of the iodine in oysters and milk are observed.

3. The response to a given dosage of iodine in the form of oysters or milk is of the same order of magnitude as that which we found in our earlier work ('33 b) for potassium iodide.

TABLE 3

*Effect of practically equivalent dosages of iodine in the form of milk and oysters*

IODINE INTAKE	TEST SOURCE OF IODINE AND AMOUNT USED IN DIET	THYROID WEIGHT	DRY MATTER IN THYROID	IODINE IN DRY THYROID
$\gamma$ per day		mg. per 100 gm. body weight	%	%
0.35	Oysters 0.86 %	$16.7 \pm 0.53$	25.2	0.038
0.37	High iodine milk 2.80 %	$15.5 \pm 0.52$	26.1	0.059
0.44	Low iodine milk 20.43 %	$15.5 \pm 0.58$	25.9	0.053
0.53	Low iodine milk 15.0 %	$18.5 \pm 0.70$	23.7	0.065
2.09	Oysters 6.45 %	$13.2 \pm 0.26$	27.0	0.114
1.98	Oysters 5.50 %	$14.1 \pm 0.54$	25.6	0.135
2.27	High iodine milk 20.43 %	$11.0 \pm 0.37$	28.7	0.113
2.14	High iodine milk 15.0 %	$13.6 \pm 0.40$	27.3	0.138

The protein of our basal diet, derived from wheat gluten and corn meal, is not of the highest biological value, and we have considered the possibility of this protein inadequacy having a part in the production of enlarged glands. This question, we believe, is answered by the results of series I and II. In series I it was necessary to feed 20% of low iodine milk in order to give about as much iodine as was contained in 2.8 % of high iodine milk. Nevertheless, the thyroid response was the same in both cases, and *corresponded to the iodine intake*. In series II, where high iodine and low iodine milk are fed at the same level, there is a marked and significant difference in thyroid response.

## THYROID SIZE AND BODY WEIGHT

Animals on the higher levels of milk and oysters made much better growth than did those on basal diet. This may have been due to improved protein, or increased mineral and vitamin supply, or both. On our GP diet growth is retarded, averaging about 10 to 12 gm. per week. When 20 % of dried milk or as little as 3 % dried oysters were added, this growth rate nearly doubled. In our goiter work heretofore we have calculated thyroid weight to 100 gm. body weight, in order to have a uniform basis of comparing different groups of animals. Growth on our diet supplemented by iodide or by very small quantities of iodine-rich foods is fairly constant, so that for such animals this method of comparison is justified. In the present work, however, rats on higher levels of milk and oysters made greater growth, reaching weights of 160 to 170 gm., instead of 120 to 130 gm., during the experimental period. This raises the question as to whether, in these cases, expressing thyroid weight in terms of 100 gm. body weight gives an accurate comparison.

According to Donaldson ('24) and McCarrison ('30), the relation of thyroid weight to body weight of normal rats between 100 and 200 gm. is nearly, but not quite linear but is not a constant. McCarrison's values, expressed in terms of 100 gm., are 9.6 mg. for 100 gm. rats, and 8.4 mg. for 170 gm. rats. Donaldson's values at both weights are almost exactly twice those of McCarrison, and in light of our experience with rats from Wistar stock, we suspect that his diet was deficient in iodine. It has not been shown that McCarrison's values will hold for rats whose growth has been retarded, as by our diet, but this seems probable. We have not as yet been able to devise a diet that will produce normal growth and at the same time be sufficiently deficient in iodine to produce consistent goiter.

We believe that for comparison of groups whose attained weight does not differ more than 20 gm., comparison on the basis of body weight is sound, but where differences as much as 50 gm. exist, some cognizance of the relatively smaller

normal thyroid size of larger animals must be taken. From McCarrison's data, the thyroid weight, per 100 gm. body weight, of 170 gm. rats, is 6.6 % less than that of 120 gm. rats; or, on our normal value of approximately 12 mg. for the smaller rats; those attaining 160 to 170 gm. weight should give 11.2 mg. The difference is not great enough to invalidate our argument.

The response to dried haddock is, if anything, superior to what one would have predicted from its iodine content. However, both levels of haddock feeding were higher than what we now consider the critical range (viz., 0.3 to 0.8  $\gamma$  per day), hence we must hesitate to say that its iodine is more adequately utilized than the other foods tested or than iodide, especially since, in this particular experiment, the response to potassium iodide was greater with respect to both thyroid size and dry matter than in the others.

Irish moss, fed at two levels, proved less effective than milk, oysters, or haddock, and less effective than we should have predicted from earlier experiments with iodide. Whether this is a peculiarity of this particular product, or points to a difference in availability of iodine from plant and animal sources, is yet to be determined.

The experiments, taken together, show with excellent agreement that, when fed at similar levels of iodine; milk, oysters and haddock possess antigoitrogenic properties of the same order of magnitude as potassium iodide. Marked advantage from either form of iodine, food or inorganic, when fed at minimal effective levels, does not appear.

#### SUMMARY

Foods rich in iodine, dried milk, oysters and haddock, and Irish moss, were fed to rats as supplements to a goiter-producing diet.

The antigoitrogenic properties of the milk, oysters and haddock are in proportion to their iodine content, and of the same order of magnitude as those of potassium iodide.

Irish moss, on the other hand, possesses lower antigoitrogenic power than is indicated by its iodine content.

The goiter-preventing properties of milk and oysters are correlated with their iodine content, and not with the improvement in protein quality or mineral and vitamin supply when they are added to a diet containing protein of poor biological quality, low in vitamins, and not adequately balanced as to minerals.

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# THE EFFECT OF THE VITAMIN A (CAROTENE) INTAKE ON THE VITAMIN D REQUIREMENT OF RATS IN THE PRODUCTION AND CURE OF RICKETS <sup>1</sup>

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A vast amount of information regarding those factors which influence the physiological processes of ossification has accumulated during the past few years. Attention has been focused primarily upon the calcium and phosphorus content of the diet and upon the presence of the antirachitic substance or substances. It has been conclusively demonstrated that certain variations among these three factors will affect the structure of bone. Little appears to be known concerning the influence of other dietary factors upon the development and cure of rickets, although there are some indications that factors other than those mentioned above may be important in this connection. This is especially true of the vitamin A content of the diet. In fact, the relationship of this dietary factor to the incidence of rickets has already attracted the attention of a few investigators. Rohmer and Dubois ('30) on conducting a series of experiments upon rachitic children receiving a minimum effective dose of vitamin D (300 to 600 rat units of viosterol per day) accompanied by daily administration of 3000 rat units of vitamin A found that such a

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<sup>2</sup> Presented by Florence S. Tabor to the faculty of the Department of Agricultural and Biological Chemistry of the Pennsylvania State College in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

treatment did not modify the action of viosterol in any way. Bacharach, Allchorne and Hazley ('31) studied the effect of adding fresh carrot to a rachitogenic diet and came to the conclusion that the addition of 1 gm. of fresh carrot daily to such a diet did not affect the rate of growth of rats, whether given in the absence or in the presence of vitamin D. Furthermore, these investigators were unable to find any correlation between the severity of rickets and the rate of growth.

Our knowledge of the marked physiological responses produced by vitamin A rich foods led us to believe that vitamin A might have some effect on the vitamin D utilization. Variations in the carotene content of yellow corn as well as in the Ca/P ratio may play a part in the variability of results which have been obtained in different laboratories. Since pure carotene is now available as a source of vitamin A and since viosterol is available as an uncontaminated source of vitamin D, it appeared desirable to study the problem further. Hence the experiments reported in this investigation were carried out for the purpose of determining whether a rachitogenic diet supplemented with different quantities of carotene, as a source of vitamin A, exhibited any influence upon the incidence and cure of rickets. The results obtained were evaluated from both the chemical and osteological findings, with attempts to correlate chemical analyses with radiographic and osteological data.

#### EXPERIMENTAL

In order to study the problem in question young growing animals of known ancestry were depleted of their body stores of vitamin D by feeding a rachitogenic diet and also by feeding a similar diet after it had been supplemented with different levels of carotene. At the end of the depletion period (twenty-first or twenty-third days) all diets were supplemented with equivalent amounts of vitamin D.

The basal diet used in these studies was the Steenbock yellow corn ration no. 2965 ('25) which consisted (in parts per 100) of yellow corn 76, wheat gluten 20, calcium carbo-

nate 3 and sodium chloride 1. Since it was desirable to maintain a definite dietary composition in order to produce a comparable degree of rickets at all times during the course of the investigation, representative samples of the basal rachitogenic ration were analyzed and found to have the following composition (in parts per 100), protein 24.26, calcium 1.52, phosphorus 0.38.

The various supplements added to the basal diet were: a) Wesson oil, b) a carotene preparation,<sup>3</sup> containing 4200 U. S. P. units<sup>4</sup> of vitamin A per gram, c) a solution of viosterol in olive oil, containing 1 U. S. P. unit of vitamin D per milligram, and d) U. S. P. reference cod liver oil, containing 95 U. S. P. units of vitamin D per gram. An attempt was also made to use a fish-oil concentrate<sup>5</sup> which contained 2,025,000 U. S. P. units of vitamin A per gram as an added source of this vitamin in these studies. The vitamin D content of this product, however, was so great that severe rickets could not be obtained when the desired levels were fed.

Rapidly growing rats 20 to 23 days old and weighing from 39 to 45 gm. each were placed and maintained in individual metal cages, which were provided with raised screen grids. Care was taken to insure uniform distribution of litters and sexes throughout the various groups of experimental animals. During the entire experimental period all animals were kept in a darkened room which was ventilated by means of an electric fan. Distilled water was kept before the animals at

<sup>3</sup> Acknowledgment is gratefully made to the S. M. A. Corporation of Cleveland for the Primatene used in this investigation.

<sup>4</sup> Guaranteed to contain not less than 3000 A. D. M. A. units per gram and converted to U. S. P. units by using conversion factor 1.4.

<sup>5</sup> Acknowledgment is gratefully made to Mr. Harden Taylor of the Atlantic Coast Fisheries, through whose courtesy a fish-oil concentrate of a very high vitamin A potency was obtained. According to Mr. Taylor this product was prepared from a halibut liver oil concentrate by molecular distillation. Previous tests by Dr. R. J. McWalter, University College, London, with the Hilger quartz echelon cell spectrophotometer gave a coefficient of absorption E of 960 at 3280A° through 1 cm. of 1 % solution. The exceedingly high potency ascribed to this product was calculated from data obtained under similar conditions on a concentrate prepared from a sample of U. S. P. reference cod liver oil (3000 units per gram).



all times. All animals were weighed at weekly intervals during the depletion period and a systematic record was made of all changes in body weight and food consumption. For the purpose of dietary treatment, these animals were arranged in experimental groups of eight to sixteen animals per group.

At the end of the depletion period and again at the end of the curative period all animals were x-rayed to determine the state of calcification. At the latter date the animals from each group were sacrificed, the blood was analyzed for calcium and phosphorus, and the long bones of the leg were used for line testing and for bone ash analyses. In the case of the blood it was necessary to pool samples from several animals of the same group, otherwise sufficient amounts were not available for analysis. Duplicate determinations were made in all cases.

To compensate for the lack of sensitivity of the radiographic examination, representative animals from the different experimental groups were killed by bleeding, as controls at various stages of the depletion period, and line tests and bone ash determinations were made of all bones obtained from such animals. In addition, the blood of these animals was used for the determination of calcium and phosphorus.

At the end of the various depletion periods, the remaining animals in all groups were continued on the respective basal diets and, in addition, were given equal amounts of vitamin D of a definite unitage. After these animals had been carried through the desired experimental period, they were killed as described above. The degree of healing or calcium deposition was measured by the x-ray and line test methods and chemical determinations were made for bone ash, blood calcium and blood phosphorus.

The right and left femora and the tibiae and fibulae were used for the line test and bone ash determinations, respectively. These bones were dissected from the animals and freed from all adhering tissue immediately after the animals were killed. The femora were removed for line tests and

photographic records, while the tibiae and fibulae were prepared for ashing by the usual extraction methods, care being exercised to prepare the bones in a uniform manner.

The proximal ends of the femora were examined at the termination of the curative period by the line test technic for evidence of recalcification. The tibiae and fibulae from each leg were wrapped in cheese cloth, dried for 24 hours in an electric oven at a temperature of 100°C., extracted continuously with hot alcohol for 8 hours, and again with ether for the same length of time. The bones were again dried to a constant weight as described above, were weighed in tared crucibles, placed in a muffle furnace and ashed for 4 hours at dull red heat. Crucibles and contents were cooled in a desiccator over night and weighed the following day. The percentage of bone ash was calculated on the basis of the dry extracted bone. Standard deviations for bone ash data were calculated.

Blood samples obtained by cardiac punctures were allowed to stand in the refrigerator over night in order that the serum might exude. The serum was pipetted off and centrifuged to free it from blood cells. An aliquot of the serum was analyzed for calcium by the Clark-Collip ('25) modification of the Kramer-Tisdall ('21) method.<sup>6</sup> The inorganic phosphorus content of the serum was determined by the Youngburg et al. method ('30).

#### DATA

An outline of the general plan of the investigation is given in table 1. In order to conserve space, summaries of data have been condensed to tabular form and are presented in tables 2 and 3.

The general plan, which was applicable to all groups in each series of experiments, consisted first in developing definite experimental rickets in animals under uniform experimental conditions by: a) Feeding rachitogenic diets to control groups, b) supplementing the basal rachitogenic diet

<sup>6</sup> The mixture was allowed to stand over night for complete precipitation of the calcium oxalate.

with definite quantities of carotene as the source of vitamin A, and c) supplementing the basal rachitogenic diet as well as the diets containing the added amounts of carotene with 5 % of Wesson oil.

It will be observed that the outline presented in table 1 has been divided into two parts, i.e., the depletion period and the curative period. It will also be observed that two sources of vitamin D were used as supplements during the curative

TABLE 1

*General outline of the experimental procedure during depletion and curative periods*

SERIES	GROUP NO.	DEPLETION PERIOD				CURATIVE PERIOD		
		Number of rats	Days on depletion	Supplements to basal rachitogenic diet		Number of rats	Days on curative	Total Vitamin D intake
				Vitamin A <sup>1</sup>	Wesson oil			
				<i>U.S.P. units</i>	<i>%</i>			<i>U.S.P. units</i>
A	1	4	21	..	..	8	18	18 <sup>2</sup>
	2	4	21	22	..	8	18	18 <sup>2</sup>
	3	4	21	90	..	8	18	18 <sup>2</sup>
B	4	5	23	..	..	5	14	14 <sup>2</sup>
	5	5	23	22	..	5	14	14 <sup>2</sup>
	6	5	23	90	..	5	14	14 <sup>2</sup>
C	7	5	23	..	..	5	12	12 <sup>2</sup>
	8	5	23	22	..	5	12	12 <sup>2</sup>
	9	5	23	90	..	4	12	12 <sup>2</sup>
D	10	2	23	..	..	5	14	1 <sup>3</sup>
	11	2	23	..	5	7	14	1 <sup>3</sup>
	12	2	23	22	5	8	14	1 <sup>3</sup>
	13	2	23	90	5	5	14	1 <sup>3</sup>
E	14	4	21	..	..	7	14	3.99 <sup>4</sup>
	15	4	21	..	5	8	14	3.99 <sup>4</sup>
	16	4	21	22	..	8	14	3.99 <sup>4</sup>
	17	4	21	22	5	8	14	3.99 <sup>4</sup>
	18	4	21	90	..	7	14	3.99 <sup>4</sup>
	19	4	21	90	5	8	14	3.99 <sup>4</sup>
F	20	6	23	..	5	8	14	3.99 <sup>4</sup>
	21	8	23	90	..	8	14	3.99 <sup>4</sup>
	22	7	23	90	5	6	14	3.99 <sup>4</sup>
G	23	6	21	..	..	11	16	...

<sup>1</sup> U. S. P. units for each gram of diet.

<sup>2</sup> Viosterol in petroleum ether.

<sup>3</sup> Viosterol in Wesson oil.

<sup>4</sup> U. S. P. reference cod liver oil in Wesson oil.

TABLE 2  
Summary of the results obtained during the depletion period<sup>1</sup>

SERIES	GROUP NO.	SUPPLEMENTS TO BASAL RADIOTOGENIC DIET		AVERAGE WEIGHT		AVERAGE DAILY FOOD INTAKE	AVERAGE DAILY VITAMIN A INTAKE	SERUM MILLIGRAMS PER 100 CC.		AVERAGE PER CENT BONE ASH	STANDARD DEVIATION
		Vitamin A <sup>2</sup>	Wesson oil	Initial	Final			Ca	P		
		U.S.P. units	%	gm.	gm.	gm.	U.S.P. units				
A	1	..	..	38.7	62.2	6.1	...	..	5.1	43.38	±0.79
B	4	..	..	42.4	63.6	5.0	...	6.8	..	33.59	±3.02
C	7	..	..	41.2	57.4	4.5	...	..	3.7	33.61	±1.21
D	10	..	..	34.5	55.0	5.5	...	..	Lost	25.80	±1.32
E	14	..	..	55.2	72.7	6.5	...	6.4	5.0	41.07	±2.59
D	11	..	5	44.5	61.0	5.5	...	..	4.8	32.48	±1.47
E	15	..	5	55.7	65.6	5.4	...	6.7	5.7	51.23	±0.23
F	20	..	5	57.6	66.6	5.7	...	6.0	4.8	41.97	±1.88
A	2	22	..	39.7	60.2	5.5	121	..	5.8	44.60	±1.17
B	5	22	..	39.2	61.2	4.9	108	6.2	..	31.14	±1.17
C	8	22	..	41.2	56.4	4.3	95	..	4.1	32.47	±2.43
E	16	22	..	48.0	66.0	5.2	114	5.2	5.8	48.34	±1.42
D	12	22	5	42.0	52.0	4.9	108	..	Lost	30.55	±3.45
E	17	22	5	45.0	63.0	4.8	106	6.8	6.1	43.35	±0.59
A	3	90	..	42.7	61.7	4.3	387	..	4.1	38.76	±0.42
B	6	90	..	44.8	68.8	5.5	495	7.0	..	30.87	±1.44
C	9	90	..	40.4	57.2	5.6	504	..	4.0	32.20	±2.17
E	18	90	..	43.0	61.0	5.2	468	5.9	5.6	43.22	±1.93
F	21	90	..	60.0	77.5	5.9	531	5.6	4.1	42.81	±3.33
D	13	90	5	40.5	54.5	5.0	450	..	5.2	29.30	±3.95
E	19	90	5	48.8	62.5	5.4	486	6.2	5.2	42.61	±1.06
F	22	90	5	54.3	63.9	5.1	459	4.8	5.2	44.04	±3.06
G	23	..	..	37.8	59.6	4.9	...	6.8	..	32.24	±2.88

<sup>1</sup> For number of rats used and length of depletion period see table 1.

<sup>2</sup> U. S. P. units for each gram of diet.

TABLE 3  
Summary of the results obtained during the curative period<sup>1</sup>

SERIES	GROUP NO.	SUPPLEMENTS TO BASAL RACHITOGENIC DIET		TOTAL VITAMIN D INTAKE	AVERAGE WEIGHT		AVERAGE DAILY FOOD INTAKE	AVERAGE DAILY VITAMIN A INTAKE	LINE TEST AVERAGE	SERUM MILLIGRAMS PER 100 CC.		AVERAGE PER CENT BONE ASH	STANDARD DEVIATION
		Vitamin A <sup>2</sup>	Wesson oil		Initial	Final				Ca	P		
		U.S.P. units	%	U.S.P. units	gm.	gm.	gm.	U.S.P. units					
A	1	..	..	18 <sup>3</sup>	66.1	74.5	5.2	...	2.5	...	4.2	48.96	±0.31
B	4	..	..	14 <sup>3</sup>	59.4	63.8	4.8	...	3.8	6.2	..	32.59	±1.26
C	7	..	..	12 <sup>3</sup>	55.0	59.6	4.5	...	2.8	...	4.9	33.97	±2.83
D	10	..	..	1 <sup>4</sup>	54.6	49.6	4.0	...	1.0	...	5.2	24.18	±5.01
E	14	..	..	3.99 <sup>5</sup>	66.5	66.0	5.0	...	1.0	11.3	6.9	36.36	±3.43
D	11	..	5	1 <sup>4</sup>	62.0	68.5	6.1	...	0.7	...	5.9	32.62	±5.87
E	15	..	5	3.99 <sup>5</sup>	57.7	58.3	3.9	...	1.3	11.4	6.0	45.63	±1.36
F	20	..	5	3.99 <sup>5</sup>	67.3	74.0	6.0	...	1.5	11.4	5.1	43.97	±8.53
A	2	22	..	18 <sup>3</sup>	58.5	68.2	4.3	95	1.7	...	3.5	47.96	±1.84
B	5	22	..	14 <sup>3</sup>	66.8	73.5	4.9	108	2.5	Lost	..	35.42	±1.40
C	8	22	..	12 <sup>3</sup>	56.2	61.8	4.2	92	2.2	...	5.0	33.02	±3.71
E	16	22	..	3.99 <sup>5</sup>	67.1	68.1	5.5	121	1.6	10.9	5.3	44.67	±1.59
D	12	22	5	1 <sup>4</sup>	65.0	70.8	5.3	117	0.9	...	7.0	32.86	±7.25
E	17	22	5	3.99 <sup>5</sup>	64.6	64.6	3.9	86	1.0	11.5	5.6	46.09	±2.37
A	3	90	..	18 <sup>3</sup>	60.4	68.5	4.4	396	2.1	...	5.1	39.63	±1.72
B	6	90	..	14 <sup>3</sup>	63.8	67.8	4.6	414	2.6	7.0	..	34.03	±4.17
C	9	90	..	12 <sup>3</sup>	57.5	64.7	4.5	405	1.5	...	6.1	32.29	±1.85
E	18	90	..	3.99 <sup>5</sup>	71.0	76.4	5.6	504	0.7	9.5	6.8	48.34	±3.30
F	21	90	..	3.99 <sup>5</sup>	70.4	81.6	6.7	603	2.3	7.4	5.6	42.78	±3.33
D	13	90	5	1 <sup>4</sup>	54.8	58.4	3.4	306	0.6	...	6.9	27.65	±8.27
E	19	90	5	3.99 <sup>5</sup>	58.5	62.0	4.1	369	0.8	6.9	5.3	42.11	±2.45
F	22	90	5	3.99 <sup>5</sup>	68.0	76.1	5.0	450	0.9	6.1	6.1	44.47	±2.40
G	23	..	..	...	41.2	68.3	5.4	...	..	10.3	3.8	27.75	±2.28

<sup>1</sup> For number of rats used and length of curative period see table 1.

<sup>2</sup> U. S. P. units for each gram of diet.

<sup>3</sup> Viosterol in petroleum ether.

<sup>4</sup> Viosterol in Wesson oil.

<sup>5</sup> U. S. P. reference cod liver oil in Wesson oil.

periods; namely, irradiated ergosterol in olive oil and U. S. P. reference cod liver oil. Either Wesson oil solutions or petroleum ether solutions of the vitamin D supplements were used. The data have been likewise summarized with reference to depletion or curative periods and are presented in tables 2 and 3, respectively.

The values for serum calcium and serum phosphorus as given in tables 2 and 3 represent group averages. Owing to limited supplies of blood serum from certain groups of animals, it was not feasible to determine both calcium and phosphorus. Degrees of healing are expressed by line test values in the conventional manner, i.e. [(—) (±) (+) (++) (+++)]. The degree of recalcification which is characterized by an unbroken thin line of darkened granules appearing on the metaphyseal side of the epiphyseal cartilage is expressed by (++), greater or lesser degrees of recalcification being designated accordingly.

In the first series of experiments (series A), three groups of animals (twelve animals per group) were used. The animals of group 1 received the unsupplemented Steenbock rachitogenic diet. The animals of group 2 received the above diet after sufficient carotene (see footnote 3) had been added to furnish 22 U. S. P. units of vitamin A for each gram of food, while the animals of group 3 received a similar diet fortified with carotene to the extent of 90 U. S. P. units of added vitamin A for each gram of food. The diets used in each series of experiments of this type were made up in quantities sufficient to complete the experiments, stored in air-tight containers, and maintained at a low temperature in an electric refrigerator. After the animals comprising this series had been on the rachitogenic diets for 21 days, four animals from each group were killed and used as controls. The remaining eight animals of each group received, in addition to the respective basal diets, 1 U. S. P. unit of vitamin D per day in the form of viosterol. The feeding of this vitamin D supplement was continued for a period of 18 days, at the end of which time the animals were killed, line tested, etc.

The animals in the second and third series of experiments (B and C) were depleted for a period of 23 days, since it appeared highly desirable to obtain a more marked degree of rickets than had been observed in series A. The curative periods were decreased to 14 and 12 days, respectively, since, in the first series (series A), recalcification was so advanced in all groups that sharp differentiation between groups was difficult.

Further variations were introduced in series D, through the introduction of a definite amount of fat in each of the above diets. In this series four groups of animals were used. The animals of group 10 received the usual rachitogenic diet, while group 11 received this basal diet after 5 % of Wesson oil had been added to it. Groups 12 and 13 received the diet containing 5 % of Wesson oil, which also contained 22 and 90 units of vitamin A per gram, respectively. The vitamin D supplement used in this series was viosterol which was made up to such a volume (in Wesson oil) that 1 cc. was equivalent to 1 U. S. P. unit. This supplement was fed in special receptacles on the first, fifth and tenth days in 0.4 cc., 0.3 cc. and 0.3 cc. portions.

In order to study the effect of the presence of fat in the diet on the vitamin A-vitamin D relationship, a fifth series of experiments was carried out. This series of experiments (series E) consisted of six groups of animals. In the beginning each group was composed of twelve animals, but unfortunately a number of these animals died during the course of the curative period. The depletion period for this series was 21 days, since radiographic examination at that time revealed that all animals showed a marked degree of rickets. The animals of this series were arranged in groups in such a manner as to make possible a comparison of the responses of those receiving diets supplemented with 5 % of Wesson oil with the responses made by other groups receiving the rachitogenic diet containing no added fat. The vitamin D supplement for this series was a solution of U. S. P. reference cod liver oil containing 95 U. S. P. units of vitamin

D per gram. This vitamin D supplement was fed in separate containers in 0.5 cc. portions on the first, fifth and tenth days. This amount of oil was equivalent to 3.99 U. S. P. units.

Series F constituted a repetition of a portion of the feeding trials of series E, i.e., of groups 15, 18 and 19. In this series the depletion period was extended to 23 days because x-ray examinations on the twenty-first day revealed that the degree of rickets manifested at that time was somewhat less than was desirable.

The animals comprising series G were used as negative controls. These animals were maintained for 37 days on the basal rachitogenic diet without additional supplement. At the end of this period, the animals were examined to determine the state of ossification, using the various criteria described elsewhere.

#### DISCUSSION

These experiments were designed primarily for the purpose of determining whether vitamin A exerts any influence upon vitamin D utilization by the rat, the vitamin A being fed at different levels, while the amount of vitamin D remained constant. While agreement of opinion regarding the cause and cure of rickets is still lacking, four methods of evaluating the results in the incidence and cure of this disorder have been quite well established; namely, x-ray, line test, bone ash and chemical analyses of the blood for inorganic constituents, especially calcium and phosphorus. These criteria have been used in an attempt to establish the etiological interdependence of vitamins A and D in the production and cure of rickets. In many instances lack of correlation between these criteria has been strikingly demonstrated.

The effect of adding carotene as the source of vitamin A to the basal rachitogenic diet may be observed from the results obtained in series A (table 2). Mild rickets was manifested by animals comprising the control group (group 1) as well as by those animals of group 2 which received the rachitogenic diet supplemented by 22 units of vitamin A.



Severe rickets was very apparent among those animals of group 3 which received the basal rachitogenic diet supplemented by 90 units of vitamin A. Similar observations were made among comparable groups of animals in series B and C. The marked similarity of results obtained from the several groups of animals comprising series D indicates that when Wesson oil was added to the diet there was no appreciable effect on the severity of rickets produced, in spite of the fact that the total gain in weight made by the animals on the low fat diet (group 10) exceeded that of all other groups of the series. A discrepancy appears in the results obtained from all groups of series E. Rickets was evident among the animals of the control group (group 14), while no indication of this disorder appeared in group 15 and only very mild rickets was observed in groups 16, 17, 18 and 19 of this series. Examination of the data does not disclose that the animals had either lost weight or were refusing food but, on the contrary, all groups had shown appreciable gain in body weight. In order to verify the results obtained in the above series, this series (series E) was repeated using three groups of animals (series F). But here again the animals of the control group (group 20) showed evidence of definite rickets, whereas those of groups 21 and 22 manifested no greater severity than had previously been shown by comparable groups in the preceding series.

In these studies x-ray technic was found to be less accurate than the osteological technic in determining the degree of rickets. It was found, however, to be sufficiently accurate to indicate that the majority of animals receiving diets supplemented with 90 units of vitamin A per gram of food manifested a more marked degree of rickets than did the control animals or those animals receiving lower levels of vitamin A.

A second criterion for estimating the intensity of rickets was the variation in bone ash values. An inspection of these data shows a serious lack of consistency. In the case of series A high bone ash values were obtained for the animals of groups 1 and 2 (43.38 and 44.60 %) and decidedly lower

figures were obtained for those of group 3 (38.76 %). Similar irregularities, although less marked, were obtained in series B and C.

There appeared to be no appreciable differences in the average bone ash values among the groups of animals comprising these two series. The bone ash values obtained in series D were noticeably lower than those which had been obtained in any of the preceding experiments. It should be noted, however, that this constituted a preliminary series in which an insufficient number of animals was used. The bone ash value for the control group (group 10) was the lowest obtained (25.80 %), although it was noted that the animals in this group made a greater increase in body weight than did the animals of the succeeding groups. It is possible to explain the low figure mentioned above on the assumption that rate of growth is recognized as a predisposing factor in the etiology of rickets. The bone ash values for the animals of the remaining groups in the series were 32.48, 30.55 and 29.30 %, respectively.

Since the data obtained in the experiment of series D indicated some tendency toward differences in bone ash values when 5 % of Wesson oil was used as an added supplement to the rachitogenic diet, it seemed advisable to repeat the experiments of series D using a larger group of animals. The data obtained in this series (series E) disclose surprisingly inconsistent results. The animals of group 14 gave an average bone ash value of 41.07 %, yet x-ray examination had indicated severe rickets in all animals of the group. The animals in group 15 manifested little or no evidence of rickets and the average bone ash value for the group was 51.23 %. In group 16 two animals gave no indication of rickets when observed radiographically, two animals showed mild rickets, and the average bone ash value for all animals of this group was 48.34 %. The bone ash values obtained for groups 17, 18 and 19 were 43.35, 43.22 and 42.61 %, respectively.

In an attempt to clarify the discrepancies just described, another experiment consisting of three groups of animals

was carried out. The three groups of animals composing this series (series F) received the same dietary treatment as those of groups 15, 18 and 19. The animals of group 20 of this series exhibited definite rickets in all cases, although the average bone ash value remained at 41.97 % or considerably lower than the value obtained in group 15 of the preceding series. Values for groups 21 and 22 were in agreement with those obtained in series E, in that there was no appreciable lowering of bone ash values although rickets was manifest to a certain degree in all animals of the series.

Examination of the data in table 2 discloses a wide variation in bone ash values. It is apparent that definite correlation does not always exist between severity of rickets (as measured by line tests) and bone ash determinations, although duplicate determinations, when made on the bones of individual animals, agree consistently. A factor which may have some bearing on the above discrepancies is the age of the test animals. Bone ash determinations on the extracted bones from normal animals of various ages gave the following values: 18 months,  $63.20 \% \pm 1.16$  (S.D.)<sup>†</sup>; 12 to 15 months old,  $56.03 \% \pm 1.36$  (S.D.); 40 to 50 days old,  $50.35 \% \pm 2.01$  (S.D.); and 30 to 40 days old,  $48.34 \% \pm 1.64$  (S.D.). The value obtained for negative controls, animals at the end of a 37-day depletion period, was  $27.80 \% \pm 2.28$  (S.D.).

Blood phosphorus values obtained in series A, C and D disclosed no significant differences. Blood calcium values obtained in series B were somewhat lower than normal values, although there was not a very great difference between individual groups of the series. The average value for the animals of the control group (group 4) was 6.8 mg. of calcium per 100 cc. of serum, while the values for the other two groups were 6.2 and 7.0 mg. per 100 cc. of serum respectively.

In the case of the animals comprising the various groups of series E and F, both blood calcium and blood phosphorus values were determined. An examination of the data in table 2 shows that the product of these values for all groups,

<sup>†</sup>S.D. (standard deviation).

except group 17, was 30 or less. Kramer, Shear and Siegel ('31) have reported that active rickets is present when this product falls below 30. The radiographic and bone ash values for these experiments, however, do not warrant such a conclusion. The addition of 22 and 90 units of vitamin A tended to lower blood calcium and blood phosphorus values, although the differences between groups were slight. Attention is called to the fact that bone ash values remained consistently high in spite of the appreciable lowering of blood calcium and blood phosphorus. In other words, there does not seem to be a definite correlation between bone ash, blood calcium and blood phosphorus values as obtained in these series of experiments during the depletion periods.

The influence of vitamin A on the vitamin D utilization and, consequently, on the cure of rickets may be deduced by likewise considering data obtained in the curative periods. An analysis of the line test data as summarized in table 3 shows that, in general, lower values for average line test healings were observed in all groups receiving vitamin A supplements than were observed among animals of the control group. The lowest values were observed among those groups receiving the basal ration supplemented with 90 units of vitamin A and 5 % of Wesson oil simultaneously. There appears to be less correlation between bone ash values and line test values at this time than there was between radiogram and bone ash values at the end of the depletion periods.

Further examination of the data discloses that wide variation in bone ash values occurred among the various groups of test animals. In series A there was no definite increase in bone ash in any of the groups over and above that observed at the close of the depletion period. Group 3, with the lowest value of 39.63 %, gave a relatively high line test average (2.1), while group 2 with a bone ash value of 47.96 % gave a line test average of only 1.7. More striking discrepancies appear among the bone ash data of series B and C; yet all of these groups gave high line test averages. Relatively low bone ash values were found for all groups of animals com-

prising series D. The lowest bone ash value was given by the control group (group 10); however, this group gave the highest line test value.

Inconsistencies in bone ash values are further emphasized in those groups comprising series E. The control group (group 14) gave a lower value for bone ash at the end of the curative period than was observed at the end of the depletion period. Similar observations are noted among the animals of groups 15 and 16. Groups 17 and 18 manifested a marked increase in bone ash values for the curative period, while group 19 showed little difference between bone ash values at the end of the depletion period and at the end of the curative period. The results obtained in series F were very comparable to those obtained in series E. On the basis of these bone ash data only indefinite information is afforded relative to the degree of recalcification. This opinion has been expressed by other investigators (Adams and McCollum, '28).

Since there appeared to be a definite lack of correlation between bone ash values and the corresponding line test averages, an attempt was made to determine the range in line test values which would result in a measurable difference in the percentage of bone ash. For this purpose the tibiae (fibulae attached) from both legs of four animals which had shown a 4(+) line test healing were removed and bone ash values were determined according to the technic previously described. The average bone ash value obtained for this group of animals was  $38.5\% \pm 3.6$  (S.D.). This same procedure was repeated using six animals, which gave a 1(+) line test healing. The average bone ash value for this group was  $33.1\% \pm 2.47$  (S.D.). In other words, a difference between a 4(+) and a 1(+) in line test values was necessary to show a difference of approximately 5% in bone ash values. It appears, therefore, that small differences in the degree of recalcification cannot be measured quantitatively by the bone ash technic.

Variations in blood calcium and blood phosphorus were obtained among those series of animals which had received vitamin D from different sources as a supplement to the rachitogenic diets. One U. S. P. unit of vitamin D daily (viosterol in petroleum ether) was the supplement used in series A, B and C. In general, it may be pointed out that very small increases in the values over those found at the end of the depletion period were manifested. Blood phosphorus values were determined for series A and C. In series A this value increased in group 3, while for groups 1 and 2 the value was lower than at the end of the depletion period. In series C all groups manifested an increase in blood phosphorus; group 9 (receiving 90 units of vitamin A per gram of food intake) gave the highest value. Blood calcium was determined for series B. The increase in this element over that observed at the end of the depletion period was very slight. In series D the supplement was 1 U. S. P. unit of vitamin D (viosterol in Wesson oil). In this series an increase in the blood phosphorus was observed among all groups of animals, the values ranging from 5.2 to 7.0 mg. of phosphorus per 100 cc. of serum.

Blood calcium and blood phosphorus values were determined in series E and F, and here some interesting observations were made. While marked increase appeared in the calcium values above those observed at the end of the depletion periods, there was no great increase in the blood phosphorus values of either series. No difference in the blood calcium value for the control group (group 14) and the group which received the rachitogenic diet plus the Wesson oil (group 15) was apparent. A similar value (11.5 mg. of Ca per 100 cc. of serum) was given by group 17, while lower values were obtained in groups 16, 18 and 19. These values ranged from 10.9 to 6.9 mg. of Ca per 100 cc. of serum.

There appeared to be but slight differences in the blood phosphorus values among the various groups of these series, the values ranging from 6.9 to 5.3 mg. per 100 cc. of serum.

The values for series F were similar to those for corresponding groups in series E. In this series a reference cod liver oil, in an amount equivalent to 3.99 U. S. P. units of vitamin D, was fed as the supplement. The average blood calcium and blood phosphorus values for groups of control animals on this supplement after a 10-day curative period was found to give 11.4 and 5.2 mg. per 100 cc. of serum, respectively.

An examination of the values obtained by the chemical analyses of the blood serum shows a lack of correlation between calcium and phosphorus values, line test values, and bone ash values. Differences of opinion and conflicting results concerning such findings appear in the literature. It has been observed by Kramer, Shear and Siegel ('31) that recalcification in rachitic bones is preceded by a rise in the blood calcium and phosphorus. Hess et al. ('30) have observed that there is a lack of correlation between healing rickets and the inorganic phosphorus of the blood and that, while in general a lowering of phosphorus takes place, this is not necessarily an essential feature of rickets.

The age of the test animal may have some bearing on the diversity of results. Analyses of blood for inorganic calcium and phosphorus from normal animals of various ages gave the following values in milligrams per 100 cc. of serum: 3 to 5 weeks—calcium 7.6, phosphorus 8.7; 90 days—calcium 9.8, phosphorus 6.6; 17 months—calcium 9.6, phosphorus 6.3; 20 months—calcium 10.9, phosphorus 6.3.

#### SUMMARY

The production of rickets in the growing rat receiving a rachitogenic diet supplemented and unsupplemented with carotene as the source of vitamin A has been studied by means of x-ray examination, line test, bone ash determinations, and by an analysis of the blood serum for calcium and phosphorus. The subsequent recalcification, when corresponding animals received such diets supplemented with vitamin D from two sources, has been investigated in a similar manner.

The manifestations of the interrelationship of vitamin A and vitamin D in the etiology of rickets depend no doubt upon several factors, among which may be mentioned 1) past ancestral and nutritional history, 2) the age of the animals, and 3) individual variation among animals of the same age. It may be observed that the values found for the percentage of bone ash for normal animals of different ages varies with age. There is also variation in the blood calcium and blood phosphorus among normal animals of different ages. It is, therefore, highly desirable that all experimental animals used in a study of this nature be from the same genetic source and be carefully standardized according to age.

The efficient conversion of carotene to vitamin A undoubtedly varies among individual animals. Consequently, a detailed study of the problem necessitates the use of a natural vitamin A concentrate free from vitamin D in order to insure a clearer understanding of the vitamin A-vitamin D relationship. Until it is possible to procure such a supplement, this interrelationship cannot be readily demonstrated.

The following observations were made during the course of the experiment:

Rachitogenic diets containing 90 U. S. P. units of vitamin A per gram of diet seemed to produce, during the depletion period, an increase in the severity of rickets among growing rats over and above that produced by a similar diet which did not contain added vitamin A supplement.

The radiographic examination at the end of the depletion period was a more reliable index of the intensity of rickets than was the percentage of bone ash.

Evidences of satisfactory correlation between the percentage of bone ash and corresponding radiograms were not obtained.

Inconsistencies between the calcium and phosphorus content of the blood serum and bone ash values were also apparent.

When equivalent unitages of vitamin D were administered, rachitogenic diets containing 90 U. S. P. units of vitamin A



per gram, with and without 5 % of Wesson oil, appeared to retard recalcification (as measured by line test technic) to a greater extent than was observed when similar diets which did not contain any vitamin A supplement were fed.

Data obtained as a result of bone ash determinations and blood serum analyses did not appear to be in agreement with line test data.

From the results obtained in this investigation the line test data appeared to be the most reliable criteria of recalcification.

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# ON THE EFFECTIVENESS OF MALT AMYLASE ON THE GASTRIC DIGESTION OF STARCHES

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This work was undertaken to determine the *in vivo* effectiveness of malt amylase on the digestion of starches. Our results bear more directly on the subject of the effectiveness of the enzyme on the gastric digestion of starches; but in addition the results show that a portion of the enzyme passes into the intestine before it is inactivated by the acid of the stomach and may aid the intestinal digestion of starches. Although salivary amylase has also been studied, most of the investigation has involved the use of malt amylase which was chosen because it is inactivated at a lower pH (pH 2.5; optimum pH 4.5) (Sherman, Thomas and Baldwin, '19) than salivary amylase (pH 4.5; optimum 6.6) and, hence, should theoretically be more effective for therapeutic use in deficiency of pancreatic amylase.

The chief specific questions we desired to answer were:

- a) How much digestion of starch actually occurs in the stomach before salivary and malt amylase is inactivated? In regard to malt amylase this question has been considered by others on the basis of *a priori* evidence and in direct experimentation. In regard to salivary amylase the observations of Bergeim ('26) on man are generally accepted. We desired more crucial experimental evidence, and to extend Bergeim's observations, using malt amylase as well as salivary amylase.
- b) How much of orally administered enzyme passes into the intestine in an active form under various conditions, such as

when the enzyme is mixed with the meal or when it is taken after a meal?

The investigation has been conducted on dogs and human subjects. The dog has served us especially because it permits the performance of crucial analytical studies, has no ptyalin in its saliva, and, hence, yields data on which to interpret the results of perhaps less crucial experiments on man.

#### CHEMICAL METHODS

*Substrate.* As a substrate we have used both 5 % cornstarch paste and a 7½ % farina (wheat) cereal paste as a test meal. The cereal was cooked 20 minutes to insure a homogeneous mixture. In certain experiments on human subjects a mixed meal was used.

*Malt amylase.* After assaying several commercial products of malt extract, a commercial preparation<sup>1</sup>, which proved to be quite uniform in activity, was chosen. One gram of this preparation, under optimum conditions of substrate and enzyme concentration and at a pH of 6.8, assayed 0.3050 Willstätter et al. ('23) amylase units. Under similar conditions but at a pH of 4.5, 1 gm. assayed 0.405 Willstätter amylase units and converted 2.0 times its weight of starch to water soluble reducing substances in 15 minutes at 37°C. Suitable control tests on the amylase content of the extract were performed (table 1) every time an experiment was made, the necessary correction for the reducing substances in the amylase preparation being made. The desired amount (vide infra) of the malt extract preparation in solution in water or milk was either mixed thoroughly with the substrate or drunk with the stomach full of food. Malt extract was chosen in preference to takadiastase because the former has a higher saccharogenic value.

The maximum amount of reducing substances calculated as maltose that could be obtained from 7.5 gm. of farina was 4.5 gm.; while 5 gm. of cornstarch (commercial) yielded 3.0 gm. of maltose. The per cent of digestion was determined on the basis of these figures which were repeatedly checked. Sufficient malt diastase was added to the test meals to give complete digestion under optimum conditions.

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<sup>1</sup> Ovaltine.

*Determination of dilution of substrate with gastric secretion.* It was necessary, in order to estimate the reducing sugars formed and present, as well as active enzyme, in the various samples obtained from the stomach, to know the degree of dilution of the substrate with gastric juice. This was

TABLE 1

*Showing the digestion of starch with and without malt amylase when introduced in an isolated pouch of the entire stomach*

OBSERVATIONS MADE	CONTROL EXPERIMENTS: NO AMYLASE ADDED			AMYLASE OR MALT EXTRACT ADDED		
	In vitro sample <sup>1</sup>	Samples taken from the stomach		In vitro sample <sup>1</sup>	Samples taken from the stomach	
		30 minutes	60 minutes		30 minutes	60 minutes
Dilution	1.00	0.88	0.72	1.00	0.86	0.82
pH	6.5	2.8	2.3	6.3	2.6	2.0
Milligrams per cent reducing substances	250	256	283	445	1043	1097
Milligrams per cent reducing substances after 1 hour of inactivation <sup>2</sup>	247	270	275	1338	1209	1230
Per cent digestion: Reducing substances found in gastric contents	8.3 <sup>1</sup>	8.5	9.4	14.8 <sup>1</sup>	34.9	36.5
Reducing substances found after incubating gastric contents for 1 hour <sup>2</sup>	8.2 <sup>1</sup>	9.0	9.2	44.6 <sup>1</sup>	40.2	41.0

The figures gives the average of three determinations on each of three dogs. Two hundred cubic centimeters of 5 per cent cornstarch continuously perfused through the stomach for 1 hour, 1 mg. of histamine being injected at the beginning of the experiment to stimulate gastric secretion. The stomach was actually perfused for 2 hours, the 1-hour figures being the more significant because of the high acidity at 1 hour prevented much further digestion.

<sup>1</sup> A control sample to determine the amount of reducing substance in test meal. This sample was not introduced into the stomach. Such a control test was made in the course of every test performed in this investigation.

<sup>2</sup> This was done to detect the presence or absence of active amylase.

accomplished by adding phenol red dye to the substrate in a known amount (2 mg. %) and determining its dilution as given below.

*Removal of non-sugar reducing substances from the samples.* It was found that the gastric secretions, as obtained under the conditions of our experiments, contained non-sugar reducing

substances, or took up some of the iodine which was the agent employed in our assay for maltose. This complicating factor was eliminated by adding an equal amount of a precipitating mixture consisting of equal parts of 10 % sodium tungstate and 1.3 N.  $\text{H}_2\text{SO}_4$ . Control determinations on such filtrates of gastric washings showed them to be free from reducing substances. Known quantities of maltose added to gastric washings, which were then treated by this method were recovered quantitatively, establishing the accuracy of the method.

*The determination.* It is imperative that the extent of starch hydrolysis be measured by a method that is reliable over a fairly wide range of values. We have investigated a number of the more commonly used methods for determining the reducing sugars resulting from the hydrolysis of starch (Schmidt, Greengard and Ivy, '34) and selected the method of Willstätter, Waldschmidt-Leitz and Hesse ('23) with certain modifications, as being best suited to our needs.

The routine for determining the maltose produced and the activity of the enzyme in a sample was as follows:

Twenty cubic centimeters of the gastric or duodenal collection is divided into two samples, A and B, of 10 cc. each. Duplicate analyses were made in the canine experiments, but could not be made in the human tests.

*Sample A* is added immediately to a test tube containing an equal volume of previously mixed 1:1 10 % sodium tungstate — 1.3 N.  $\text{H}_2\text{SO}_4$  which has stood for 5 or 10 minutes. This procedure insures a more complete precipitation of the non-sugar reducing substances and insoluble colloidal starch. The contents of the tube are intimately mixed and the tube is then set aside while the remaining collections are accumulated for analysis.

*Sample B* is placed in a constant temperature bath ( $37^\circ$ ) and allowed to incubate for 1 hour, after which it is inactivated by treating with the precipitating mixture by the same technic used with sample A. Sample B is so treated to ascertain the amount of active diastase that passed through the stomach into the duodenum, or the amount of active enzyme in the gastric sample.

Both series of tubes (A and B) are then centrifugalized and a 5 cc. portion of the supernatant fluid from each is transferred into 250 cc. Erlenmeyer flasks. These 5 cc. portions are used for the sugar determinations. Other 5 cc. portions of the 'A' series are pipetted into test tubes for the determination of phenol red concentration. To each of the latter is added 1 cc.

of concentrated NaOH. The resulting purplish-red color is compared in a colorimeter against an identically treated portion of the original cereal test meal which serves as the standard. Diluting the standard to one-fourth or one-half its original concentration gives a standard that matches the unknown samples closely and within the range of optimum accuracy. The values expressed in our tables under the heading 'dilution' represent the percentages of dye in the diluted gastric samples as compared to the original concentration of the test meal when introduced into the stomach.

To the fluid in each Erlenmeyer flask are added 10 cc. N/10  $\text{KI}_3$  and the mixture is then made alkaline by slowly adding, with shaking, N/8 NaOH. About 50 cc. of the alkali are required to convert the iodine to sodium hypoiodite which is colorless; however, due to the presence of the phenol red dye the contents of the flask appear pink when this point is reached. The flask is then allowed to stand 15 minutes after which 2 cc. of 10 %  $\text{H}_2\text{SO}_4$  are added. The sample is then titrated to a colorless (yellowish) end point with N/20  $\text{Na}_2\text{S}_2\text{O}_4$ . One cubic centimeter of N/10  $\text{KI}_3$  is equivalent to 17.15 mg. of maltose so long as the volume of iodine taken up is between 0.5 and 3.5 cc. Above a titration value of 3.5 cc. of  $\text{KI}_3$  a straight line relationship no longer exists for the  $\text{KI}_3$ -maltose ratio. In order that the existing curvilinear relationship be taken into consideration, we have plotted a curve based upon the iodine taken up by known amounts of maltose varying from 10 to 100 mg. Titration values on routine determinations are referred to this curve for maltose equivalents.

The reducing sugar is expressed in terms of milligram maltose per 100 cc. of the original substrate by the following calculation:

$$\frac{\text{Mg. maltose in 5 cc.} \times 40^3}{\text{Per cent of original concentration.}} = \text{Mg. per cent maltose}$$

(from phenol red determination)

It has been pointed out that 7.5 gm. of farina yielded 4.5 gm. of maltose. The value mg. per cent maltose is calculated to per cent digestion by dividing by 4500 mg. The difference between the values in samples A and B indicates the presence or

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<sup>3</sup> Five cubic centimeters, taken for sugar determination, multiplied by four equals the amount in the 20 cc. of treated gastric or duodenal sample. This value represents the amount of reducing sugar in 10 cc. of the cereal substrate, therefore, the amount in 5 cc. times four times ten equals milligram maltose in 100 cc. of the diluted sample. Dividing by the concentration of the diluted sample gives the milligram maltose per 100 cc. in terms of the original concentration.

absence of active amylase. This difference may be expressed in terms of Willstätter units by substituting the appropriate values into the formula  $K = \frac{1}{t} \log \frac{a}{a-x}$  (Willstätter, et al., '23).

#### PHYSIOLOGICAL METHODS

##### *In dogs*

Digestion of starch in the stomach and the degree of activity of the amylase administered was studied by three different physiological techniques, which supplied crucial data.

A. *Dogs with a pouch of the entire stomach.* Three dogs with a pouch of the entire stomach were employed. In these under aseptic conditions the stomach was isolated from the alimentary tract. The cardiac end of the stomach was closed and the pyloric end opened to the outside by a fistula, the integrity of the alimentary tract being restored by an esophago-duodenal anastomosis (Lim, Ivy and McCarthy, '25). These animals, of course, remain in excellent condition for years. With this preparation it was possible to place the substrate with or without malt extract into the stomach within a period of 5 minutes and study digestion without possible contamination with intestinal secretions. Further, the gastric glands of the pouch could be stimulated with histamine, so as to follow the effect of pH on the course of the digestion. In these animals 200 cc. of 5 % starch paste with or without enzyme was placed in the stomach and histamine (1 mg.) was injected. Samples of the substrate were removed at  $\frac{1}{2}$ -hour intervals from maltose and enzyme assay.

B. *Dogs with a complete duodenal fistula, exclusion of bile and pancreatic juice, and jejunostomy.* Three dogs were prepared as follows: The pancreas was separated from the duodenum to exclude pancreatic secretion. The common bile duct was tied and cut and the gall bladder anastomosed to the ileum, thus excluding bile from the duodenum. This was done at a preliminary operation. At a second operation the duodenum was sectioned 3 inches below the pyloric sphincter, and the distal end was closed and the proximal end converted into a fistula. A jejunostomy was then performed, through which the animal was fed by a technic previously used in this laboratory (Scott and Ivy, '31). The farina meal with or without enzyme was either fed by mouth or introduced via gastrostomy, from 3 to 5 minutes being required to get the meal into the stomach. In this preparation the entire gastric discharge was collected from the duodenal fistula. Saliva, of

course, was swallowed but it is well known that dog's saliva contains only traces of diastase at the most. A small amount of duodenal secretion was probably present in the chyme collected from the duodenal fistula but no pancreatic juice and bile. We, thus, had an almost perfect test animal for these studies.

C. *Dogs prepared as in B, having a gastric fistula, but with an incomplete duodenal fistula through which duodenal chyme was collected by a catheter.* Four such animals were prepared because it is easier to preserve excellent health in them. By means of the phenol red determinations it was shown in each experiment on these animals that we were able to recover through the catheter (24 French) from 40 to 80 % of the chyme emptied from the stomach.

### *Human subjects*

Graduate medical students were used as subjects. The experimental methods will be given later. Determinations of reducing sugar, active amylase present in gastric contents, dilution and acidity were made on the gastric samples. In addition, tests to correlate gastric emptying time and acidity were made.

### RESULTS

*Total gastric pouch dogs.* Six tests were made on each of the three dogs. The introduction of 5 % starch paste without malt extract served as a control for the introduction of the starch paste with malt extract (0.35 gm. desiccated malt extract). The difference between the reducing sugar values served as an index of the extent of enzyme action.

The entire data on starch digestion in the stomach pouches are too bulky to submit; yet they were so consistent, even between different dogs, as to permit the presentation of averages. This is done in table 1. Referring to the table, it is to be noted that the acidity increased and that dilution resulted. The addition of malt extract increased the per cent of digestion of starch, the average at 1 hour being 36.5 %. Ninety-five per cent of this digestion occurred within the first 30 minutes, or during the time the pH of the gastric contents changed from a pH of 6.3 to 2.6. This is due to the fact that the amylase in the malt extract we used is inhibited when the pH falls below 4.0 and inactivated at pH 2.5.



*Complete duodenostomy dogs.* Both the cornstarch and cereal test meals were used. The cornstarch meal was soon discarded because it was liquefied and evacuated from the stomach so rapidly. At 1 hour, when the stomach was about empty, 60 % of the starch had been digested and very little of the enzyme had been destroyed. The cereal meal (amount varied according to the size of the dog) was not evacuated

TABLE 2

SAMPLE	VOLUME COLLECTED	VOLUME COLLECTED CORRECTED FOR DILUTION	ACID			DILUTION	MILLIGRAM PER CENT MALTOSE		GRAMS MALTOSE RECOVERED	PER CENT DIGESTION	
			pH	Free Cl. U.	Total Cl. U.		At recovery	After incubation to determine active enzyme present		At recovery	After incubation

Dog 3. 'Starch digestion' in complete duodenostomy dogs. 400 cc. 7½ % farina cereal. No malt extract added. Recovered 265 cc. (corrected volume) in 60 minutes. Average of three determinations

15 minutes	90	78	4.9	5.0	12.5	0.87	408	415	0.32	9.1	9.2
30 minutes	110	90	4.1	10.0	15.0	0.82	420	490	0.38	9.3	10.9
45 minutes	75	55	3.7	12.0	20.0	0.73	515	520	0.28	11.4	11.5
60 minutes	60	42	3.2	15.0	25.0	0.70	556	572	0.23	12.4	12.7
Duodenal collection		265							1.21		
Left in stomach		135							0.74		
Total		400							1.95	10.8	

Dog 3. Effect of malt amylase on starch digestion. 400 cc. 7½ % farina cereal containing 0.70 gm. malt extract. Recovered 316 cc. (corrected volume) in 60 minutes. Average of three determinations

Control <sup>1</sup>			6.8			1.00	582	1970		12.9	43.8
15 minutes	111	99	5.2	5.0	10.0	0.89	1470	1837	1.45	32.7	40.8
30 minutes	160	136	4.8	6.5	12.5	0.85	1744	2210	2.37	38.8	49.2
45 minutes	85	66	4.4	7.5	15.0	0.78	2260	2351	1.49	50.3	52.3
60 minutes	20	15	3.6	10.0	17.5	0.74	2591	2502	0.39	57.6	55.7
Duodenal collection		316							5.70		
Left in stomach		84							2.18		
Total		400							7.88	43.8	

<sup>1</sup> Not introduced into stomach; in vitro test.

so rapidly, but the most variable factor observed in these experiments was the rate of emptying of the stomach. The individual experiments were not continued until the stomach was completely empty, but until the pH fell to a point at which the enzyme could no longer act. This generally in these dogs required 1 to 1½ hours. The maltose remaining in the stomach could then be easily removed via the gastric fistula, assayed, and the total maltose produced by the amylase calculated.

The results of a typical experiment on one dog is shown in table 2. For comparative purposes only the data obtained

TABLE 3  
*Summary of data on three complete duodenostomy dogs*

DOG NO.	COLLECTED FROM DUODENUM		VOLUME LEFT IN STOMACH (CORRECTED)	pH CHANGE	POTENTIAL MALTOSE INTRODUCED IN FORM OF STARCH (GRAMS)	MALTOSE RECOVERED (GRAMS)			PER CENT OF STARCH CONVERTED TO MALTOSE <sup>1</sup>	PER CENT OF ACTIVE ENZYME RECOVERED IN INTESTINE
	Volume in 1 hour <sup>1</sup>	Corrected volume (dil. x vol.)				From duodenum	Stomach	Total		
1	360	340	60	7.0-5.2	18.0	7.1	1.6	8.7	48.3	85.0
2	375	331	69	7.0-4.8	18.0	9.8	2.1	11.9	66.0	82.7
3	376	316	84	6.8-3.6	18.0	5.7	2.2	7.9	43.8	79.0

Average of all tests. 400 cc. 7½ % farina cereal containing 0.7 gm. malt extract.

<sup>1</sup> One hour arbitrarily chosen to compare results.

<sup>2</sup> This value represents total conversion of starch to reducing sugars during 1 hour in vivo. It differs from 'per cent digestion,' because the latter represents the condition present at the time the sample was taken and can be used for comparative purposes only.

during the first 60 minutes of the test are given. All the data are summarized in table 3.

It was noted that the average emptying time of the cereal meal without enzyme was approximately 80 minutes, whereas with the enzyme the emptying time averaged a little more than 60 minutes. This was obviously due to the liquefying action of the enzyme.

The amount of digestion of the cereal without added enzyme varied from 5 to 12 %, with the added enzyme from 30 to 65 %, the average being 52 %. The higher percentages of digestion,

of course, occurred in those experiments in which the pH of the gastric contents fell more slowly. From 79.0 % to 85.0 %, average 82.2 % of the malt amylase passed into the duodenum without being inactivated. The per cent of digestion was greater in these than in the gastric pouch experiments, because of the higher acidity obtained by histamine injection in the latter.

Two experiments were performed in which the subject was permitted to ingest the cereal meal and then a known quantity of a solution of malt extract in water was drunk by the dog. The discharge from the duodenal fistula was collected and analyzed for amylase to determine the amount that passed. It was found that approximately 75 % of the enzyme solution drunk was passed into the duodenum within 10 minutes; the remainder mixed with the gastric contents. (See Ivy, '18, for evacuation of fluids with food in the stomach.) In these instances the gastric contents showed from 16 to 20 % digestion of the test meal at the end of 1 hour. These results, when compared to the above results, show that the quantity of enzyme that remained in the stomach was not adequate for the digestion of the starch and that when it is desired to have active amylase pass through the stomach rapidly the enzyme should be taken after a meal.

*Duodenal and gastric fistula dogs, the duodenal chyme being collected in part by a catheter.* In the preceding experiments the dogs were so prepared as to obtain unequivocally the total discharge from the stomach. The four dogs used in the following experiments were easily maintained in excellent health for months. Although the total gastric discharge could not be collected through the catheter (24 French) in the duodenum, from 40 to 80 % of the discharge could be collected and the total discharge calculated from the phenol red determinations. The gastric fistula rendered the determination of the emptying time of the stomach very accurate. Such dogs as a rule do not empty their stomach as rapidly as a dog with a complete duodenal fistula. The enterogastric reflex does not operate well in the latter animals.

The results obtained compare very favorably with the results on the complete duodenostomy dogs. The effect of malt extract on gastric emptying was the same. The total amount of gastric starch digestion was somewhat less on the average varying from 30 to 40 %. This was due to the fact that the acidity of the gastric contents in these dogs rose more rapidly as a rule. However, 87.5 % of active amylase passed into the duodenum. The average results on the dog which showed the least digestion due to a more rapid rise in acidity are shown in table 4.

TABLE 4  
'Starch digestion' in duodenal fistula dogs

SAMPLE	VOLUME COLLECTED CORRECTED FOR DILUTION	ACID			DILUTION	MILLIGRAM PER CENT MALTOSE		GRAMS MALTOSE RECOVERED	PER CENT DIGESTION	
		pH	Free Cl. U.	Total Cl. U.		At recovery	After incubation to indicate presence of active enzyme		At recovery	After incubation to determine amount of enzyme present
Dog no. 5. 400 cc. 7½ % farina cereal. No malt extract added. Recovered 184 cc. (46 %). Emptying time 80 minutes. Average of three determinations										
Control		7.0			1.00	204	280		4.5	6.2
30 minutes	64	4.3	3.5	11.0	0.83	277	490	0.18	6.2	10.9
60 minutes	109	3.2	7.0	18.0	0.69	480	512	0.52	10.6	11.4
Duodenal collection	173							0.70		
Into intestine	195							0.93		
Left in stomach	32							0.07		
Total	400							1.70	9.4	
Dog no. 5. 400 cc. 7½ % farina cereal containing 0.7 gm. malt extract. Recovered 258 cc. (64.5 %). Emptying time 55 minutes. Average of three determinations										
Control <sup>1</sup>		7.0			1.00	592	2030		13.2	45.0
10 minutes	95	4.8	7.5	13.5	0.79	932	1665	0.88	20.7	37.0
20 minutes	67	4.2	6.5	12.5	0.67	1670	2840	1.12	37.0	63.2
40 minutes	56	3.0	10.0	20.5	0.62	1733	2063	0.97	38.6	46.0
55 minutes	40	2.4	29.0	33.5	0.64	1780	1767	0.71	39.5	39.2
Duodenal collection	258							3.68		
Into intestine	136							1.94		
Left in stomach	6							0.02		
Total	400							5.64	31.3	

<sup>1</sup> Not introduced into stomach.

*Comparison of human saliva with malt diastase.* Two experiments were performed in which an amount of human saliva diastatically equivalent to the amount of malt diastase employed was added to the farina. One cubic centimeter of the human saliva used was equivalent to 140 mg. of the malt

TABLE 5

*Effect of human saliva on starch digestion in duodenal fistula dog*

Dog no. 5. 400 cc. 7½ % farina cereal containing 5 cc. human saliva. Recovered 214 cc.; emptying time 45 minutes; average of two determinations

SAMPLE	CORRECTED VOLUME	ACID			DILUTION	MILLIGRAM PER CENT MALTOSE			GRAMS MALTOSE RECOVERED	PER CENT DIGESTION		
		pH	Free Cl. U.	Total Cl. U.		At recovery	After incubation	Reactive to pH 7.0 and incubated at 37°		At recovery	After incubating	Attempting to reactivate by readjusting to pH 7.0 and incubation at 37°
Control <sup>1</sup>		6.8			1.00	548	1784			12.2	39.7	*
10 minutes	75	5.8	....	10.0	1.00	784	1840	1910	0.59	17.4	40.9	42.4
25 minutes	52	5.9	....	7.5	0.92	1270	2260	2260	0.66	28.2	50.3	50.3
35 minutes	74	4.6	4.6	15.0	0.78	1320 <sup>2</sup>	1420	1400	0.98	29.3 <sup>3</sup>	31.5	31.1
45 minutes	13	2.9	35.0	55.0	0.63	1230	1240		0.16	27.3	27.6	
Duodenal collection	214								2.39			
Into intestine	171								1.91			
Left in stomach	15								0.18			
Total	400								4.48	24.9		

<sup>1</sup> Enzyme inactivated at this point.

<sup>2</sup> Enzyme inhibited at this pH.

<sup>3</sup> Not introduced into stomach.

<sup>4</sup> Figures in this column show that adjusting the pH of the duodenal contents to neutrality does not reactivate the inactivated ptyalin.

extract (comparisons made at pH 6.8). The results are shown in table 5, and they may be compared to the results in table 4, which were obtained using malt extract. It is to be noted that less maltose (approximately 20 %) was obtained with the diastatically equivalent amount of saliva. This was due to the fact that the ptyalin was inactivated at pH 4.5, and

malt extract acts longer in the stomach. Thirty-one and seven-tenths per cent of the active ptyalin passed into the duodenum during the first 25 minutes or before the acidity rose to the point of inactivation of ptyalin.

*Is salivary or malt amylase inactivated by acid in the stomach reactivated in the duodenum?* This was investigated because of Roger's ('07) claim that saliva inactivated by acid may be reactivated by traces of amylase. Bergeim ('26) found this not to be the case. Our results confirm Bergeim, who pointed out that Roger failed to control the electrolyte concentrations of his digestion mixtures.

*Amount of malt amylase that passes into the intestine when ingested in milk and water with food in the stomach.* On the complete duodenal fistula dogs cited above, two experiments were done in which it was found that when malt extract is ingested in water after a meal 75 % of the active enzyme passed into the duodenum within 10 minutes. Since, as is well known, milk does not pass into the duodenum so rapidly, it was decided to compare milk with water in two dogs with an incomplete duodenal fistula, an animal preparation which is more normal in regard to gastric evacuation than the former. Further, it was thought that the acid-buffering properties of the milk would retard the rise in pH of the gastric contents and prolong the action of the amylase in the stomach.

Three tests were performed on each of two dogs as follows: Five minutes after the ingestion of the cereal meal, 250 cc. of either milk or water containing 0.7 gm. of malt extract and the phenol red indicator was drunk by the dogs. The assay had to be modified to consider the reducing substances present in the milk. Continuous collections from the duodenal fistula were made, the various assays being made for enzyme, dilution, etc., at 15-minute intervals. With water the quantity of enzyme that passed into the duodenum during the first 30 minutes varied between 62 and 71 %. With milk during the same period from 34 to 54 % of enzyme was recovered from the duodenum. The acidity of the gastric contents rose

more slowly with milk than with water. As a result slightly more gastric digestion of starch occurred in the milk than in the water tests. The results of one of the tests are shown in table 6.

These results show again that if it is desired to have the enzyme pass through the stomach into the intestine rapidly, it is best to ingest the enzyme in water after eating a meal.

TABLE 6

*Starch digestion and active enzyme recovered from intestine of duodenal fistula dog*  
400 cc. 7½ % farina ingested followed by drinking 250 cc. water or milk containing dye and 0.7 gm. malt extract

DOG NO. 15	VOLUME		PH CHANGE	POTENTIAL MALTOSE INTRODUCED IN FORM OF STARCH (GRAMS)	MALTOSE RECOVERED (GRAMS) FROM DUODENUM	PER CENT OF STARCH CONVERTED TO MALTOSE	PER CENT OF ACTIVE ENZYME RECOVERED FROM DUODENUM
	Collected from duodenum (cc.)	Corrected volume (for enzyme solution)					
Water							
At 30 minutes	235	157	6.5 -4.25	18.0	3.37	18.7	62.8
At 60 minutes	420	218	4.25-3.25		5.53	30.8	87.2
Milk							
At 30 minutes	215	135	6.5 -5.1	28.0 <sup>1</sup>	5.04	18.0	54.0
At 60 minutes	330	207	5.2		8.97	32.0	83.0

<sup>1</sup> In farina 18.0 gm.; in milk — 10.0 gm.

Of course, if an excess of malt amylase is taken either in water or milk, a sufficient quantity will remain in the stomach to effect complete or almost complete gastric digestion of starch.

#### EXPERIMENTS ON HUMAN SUBJECTS

The experiments on the dogs were conducted primarily to obtain more crucial data upon which to base the interpretation of possibly less crucial data on human subjects. In the human subject such experiments are further complicated by the presence of salivary amylase, a factor that cannot be entirely controlled. Only graduate students in physiology accustomed to swallowing the gastric tube were employed so

that in some experiments an attempt to control the salivary factor might be made. The various assays made in the experiments on the dog, except those on duodenal chyme, were made on the gastric contents of the human subjects, samples being withdrawn in some instances and all of the contents in others.

*Experiments in which only a limited quantity of saliva was swallowed.* Tests were performed on eight subjects in which

TABLE 7

*Gastric digestion of starch in man (rapid increase in acidity) subject J.G.*

400 cc. 7½ % farina cereal. Without and with malt extract

Stomach evacuated and lavaged with acid prior to the digestion of a meal, the saliva formed after the ingestion of a meal being expectorated

SAMPLE	VOLUME COLLECTED (cc.)	CORRECTED VOLUME	ACID			DILUTION	MILLIGRAM PER CENT MALTOSE		PER CENT DIGESTION	
			pH	Free Cl. U.	Total Cl. U.		At re-covery	After in-cubation	At re-covery	After in-cubation
Control test—no malt extract										
In vitro <sup>1</sup>			6.5			1.00	584	856	13.0	19.0
15 minutes	30	26	2.7	15.0	25.0	0.87	856	984	19.0	21.8
30 minutes	30	19	1.9	45.0	52.5	0.65	1140	1310	25.4	29.2
45 minutes	30	14	1.8	45.0	53.7	0.48	1240	1220	27.6	27.1
With malt extract (0.7 gm.)										
In vitro control <sup>1</sup>			6.7			1.00	440	2200	9.8	48.8
10 minutes	30	32	3.8	5.0	12.5	0.75	1865	3270	41.6	72.6
20 minutes	30	18	2.2	17.5	30.0	0.61	2000	2170	44.4	48.3
30 minutes	30	16	1.8	25.0	35.0	0.55	2000	2125	44.4	47.3
45 minutes	30	15	1.4	55.5	72.5	0.51	1995	2105	44.3	46.8

<sup>1</sup> Not introduced into stomach.

the cereal meal (400 cc. 7.5 % farina) was subjected only to that amount of saliva swallowed during the ingestion of the meal, which required from 3 to 5 minutes, the malt extract being mixed with the meal just immediately before its ingestion. Prior to the ingestion of the meal the stomach was evacuated of its contents and then lavaged with 0.2 % HCl to inactivate any ptyalin present. After the ingestion of the meal the subject expectorated the saliva secreted. This was done to simulate a deficiency of salivary ptyalin.



Samples of the gastric contents were withdrawn at 10- or 15-minute intervals and assayed for reducing sugar, pH, enzyme, etc.; the phenol red indicated the dilution factor. One test was made with and one without adding malt extract. The response of three of the eight subjects is shown in tables 7, 8 and 9. The response of the eight subjects are summarized

TABLE 8

*Gastric digestion of starch in man (moderate increase in acidity) subject J.B.*  
400 cc. 7½ % farina cereal. Without and with malt extract

SAMPLE	VOLUME COLLECTED	ACID			DILUTION	MILLIGRAMS MALTOSE PER CENT		PER CENT DIGESTION	
		pH	Free acid	Total acid		At re- covery	After in- cubation	At re- covery	After in- cubation
Without malt extract									
In vitro control <sup>1</sup>		7.0			1.00	272	240	6.1	5.3
10 minutes	40	5.9		8.0	1.20	960	1905	21.3	42.3
20 minutes	40	2.4	37.5	50.0	0.83	1240	1290	27.5	28.7
30 minutes	40	2.0	47.5	65.0	0.92	1190	1230	26.4	27.3
45 minutes	40	1.9	60.0	80.0	0.80	1170	1290	26.0	28.7
With malt extract (0.7 gm.)									
In vitro control <sup>1</sup>		6.5			1.00	652	3400	14.4	75.5
10 minutes	40	6.3		7.5	0.68	2720	4540	60.3	100.0
20 minutes	40	3.2	20.0	37.5	0.65	2860	2960	63.5	65.8
30 minutes	40	2.6	45.0	62.5	0.42	3430	3430	76.0	76.0
45 minutes	40	2.4	42.5	57.2	0.49	3100	3100	69.0	69.0
50 minutes	40	2.3	52.5	67.5	0.46	3130	3130	69.6	69.6

<sup>1</sup> Not introduced into stomach.

in table 10. In giving the per cent of digestion only the amount of gastric digestion of starch found is considered, because the amount of undigested starch that passed into the intestine was not and could not be accurately ascertained in normal subjects.

It was found that the amount of gastric starch digestion depended on the rate at which the acidity of the gastric contents rose, more starch digestion being obtained when malt extract was added in seven out of the eight subjects. The responses may be divided into three or perhaps four groups. A) In subjects 1, 2 and 3 the acidity rose rapidly to the point of inactivation of ptyalin, but not malt amylase. In subject 4,

TABLE 9

*Gastric digestion of starch in man (delayed increase in acidity). Subject E.S.*  
400 cc. 7½ % farina cereal. Without and with malt extract

SAMPLE	VOLUME COLLECTED (CORRECTED FOR DILUTION)	VOLUME INTO DUODENUM	ACID			DILUTION	MILLIGRAMS MALTOSE PER CENT		PER CENT DIGESTION	
			pH	Free acid	Total acid		At recovery	After incubation to detect enzyme present	At recovery to defect amount of enzyme present	After incubation
Without malt extract										
In vitro control <sup>2</sup>			6.5	...	...	1.00	178	178	3.9	3.9
15 minutes	242	158	5.4	...	5.0	0.90	3290	4030	73.0	89.5
30 minutes	42	316	5.3	...	5.0	0.87	3800	4020	84.5	89.3
45 minutes <sup>1</sup>										
With malt extract (0.7 gm.)										
In vitro control <sup>2</sup>			6.5	...	...	1.00	712	1950	15.8	43.4
10 minutes	284	116	4.9	...	5.0	1.00	3090	5290	68.5	100.0
20 minutes	170	206	5.5	...	5.0	0.83	4300	5110	95.5	100.0
30 minutes	60	291	5.6	...	5.0	0.71	5100	5360	100.0	100.0
45 minutes <sup>1</sup>										

<sup>1</sup> Stomach empty.

<sup>2</sup> Not introduced into stomach.

the same type of response occurred, but the acidity rose somewhat more slowly yielding 36 % digestion with ptyalin and 78 % with malt amylase. B) In subject 5 the acidity at 45 minutes was as high as in those in the first group, but it rose initially more slowly, which accounts for the marked digestion with both the salivary and malt amylase. C) In subject 6 the acidity rose very slowly and the stomach emptied

rapidly which accounts for high values for gastric starch digestion. D) In subjects 7 and 8 the acidity rose to the lethal point of ptyalin and malt amylase at about the same time, so that malt amylase did not increase the gastric digestion of starch.

The early part of the curve of the rate of digestion in the salivary and malt amylase tests are quite identical. The malt amylase simply continues to act from 5 to 15 minutes longer in the stomach than salivary amylase.

TABLE 10

*The effect of malt amylase on the gastric digestion of starch in man*

Values represent the conditions present 45 minutes after ingestion of 400 cc. 7½ % farina cereal, with and without the addition of 0.7 gm. malt extract

SUBJECT	CONTROL			WITH MALT EXTRACT		
	pH	Per cent digestion at recovery	Active ptyalin	pH	Per cent digestion at recovery	Active amylase
1. (J.G.)	1.8	27.6	Absent	1.4	44.3	Absent
2. (J.B.)	1.9	26.0	Absent	2.4	69.0	Absent
3. (W.B.)	1.6	26.4	Absent	1.3	68.2	Absent
4. (J.V.)	4.1	36.7	Absent	4.0	78.5	Present
5. (R.B.)	2.0	73.4	Absent	2.1	98.0	Absent
6. (R.S.) <sup>1</sup>	5.3	84.5	Trace	5.6	100.0	Present
7. (D.M.)	3.0	54.8	Absent	1.5	58.2	dig. compl.
8. (R.R.)	2.2	29.7	Absent	1.9	26.1	Absent
Approximate average		45			68	

<sup>1</sup> Stomach empty at 45 minutes, values given for 30 minutes.

In order to obtain more accurate data pertaining to the amount of active enzyme that passed into the duodenum, five of the subjects were given the meal under the above conditions and 10 or 15 minutes later the stomach was completely evacuated. A sample was kept for assay and the remainder injected into the stomach to be removed again 10 or 15 minutes later. The data are summarized in table 11 and show that a minimum of 42 % of the enzyme had passed into the duodenum before inactivation. In the hypochlorhydric subject 'R. S.' approximately all entered the duodenum in active

form. The average for the five subjects was 54 % which is less than in the experiments on dogs, because the acidity on the average rose more rapidly in our human subjects.

*Human subjects: Experiments in which saliva was swallowed at all times.* These experiments were performed to simulate normal conditions of eating. In the first experiment

TABLE 11  
*Summary of results on human subjects*

SUBJECT	PH CHANGE DURING TEST PERIOD	VOLUME AT END OF TEST PERIOD		PER CENT DIGESTION OF STARCH IN STOMACH AT END OF TEST PERIOD	AVERAGE PER CENT DIGESTION OF STARCH ENTERING DUODENUM DURING TEST PERIOD	MALTOSE (GRAMS)			TOTAL PER CENT STARCH CONVERTED TO MALTOSE	PER CENT ACTIVE ENZYME ENTERING DUODENUM
		In stomach	Passed into duodenum			Recovered	Entered	Total		
J.B. (15)	6.5-3.2	282	118	60	37	7.62	1.97	9.59	53.2	29.5
.... <sup>1</sup>										
D.M. (15)	6.5-3.2	230	170	30	20	3.11	1.53	4.64	25.8	42.5
.... <sup>1</sup>										
R.S. (10)	6.5-5.6	284	116	69	42	8.83	2.19	11.02	76.9	90.0
(20)		141	259	96	83	6.10	7.52	13.62		
(30)		43	359	100	98	1.90	11.94	13.84		
R.R. (15)	6.0-2.6	150	250	23	18	1.55	2.02	3.57	19.8	62.5
.... <sup>1</sup>										
W.B. (15)	6.0-2.7	215	185	45	29	2.81	2.19	5.00	27.8	46.2
.... <sup>1</sup>										
								Average	54.3	

Results based on the gastric starch digestion that occurred during the time that the pH was falling to a level near that at which malt amylase is inactivated (pH 2.4). In these experiments the stomach was entirely evacuated every 10 or 15 minutes, a sample taken for assay and the balance returned depending on the pH found.

<sup>1</sup> Data for other 15-minute periods not given because point of inactivation of enzyme has been reached prior to 20 minutes.

of this type the cereal meal with and without malt extract was ingested and all the saliva was swallowed, the malt extract being mixed with the cereal just before it was ingested. Only one experiment of this kind was done because the results were the same as in the above experiments on this subject, i.e., more starch digestion occurred when malt extract was

added. This was due to the fact that the acidity of the gastric contents destroyed the ptyalin of the swallowed saliva.

The following experiment was then performed on ten human subjects, eight of whom had been used in the above experiments. The test meal used consisted of 200 gm. of mashed

TABLE 12

*Starch digestion in the human stomach when a mixed meal is ingested with and without the addition of malt amylase to milk consumed at intervals during the meal. Stomach completely evacuated at end of 2 hours*

SUBJECT	PH AT END OF 2 HOURS	CLINICAL UNITS OF HCl		PER CENT DIGESTION OF STARCH	PRESENCE OR ABSENCE OF ACTIVE ENZYME IN STOMACH
		Free	Total		
Without malt amylase	4.10	32.5	62.5	92.3	Absent
J.B. With malt amylase	4.18	21.5	51.2	100.0	Present
Without malt amylase	4.10	32.5	67.5	85.7	Absent
W.B. With malt amylase	4.43	20.0	47.5	100.0	Present
Without malt amylase	3.70	40.0	67.5	100.0	Absent
R.R. With malt amylase	3.80	42.0	47.5	100.0	Present
Without malt amylase	4.26	22.5	62.5	100.0	Absent
L.L. With malt amylase	4.30	27.5	60.0	100.0	Present
Without malt amylase	2.82	65.0	115.0	100.0	Absent
R.B. With malt amylase	2.90	62.5	112.0	100.0	Absent
Without malt amylase	4.4	5.0	20.0	72.0	Present
M.P. With malt amylase	4.8	12.0	30.0	100.0	Present
Without malt amylase	3.0	70.0	112.5	94.2	Present
M.D. With malt amylase	2.9	68.0	115.0	95.0	Present
Without malt amylase	3.1	67.5	100.0	98.5	Absent
J.G. With malt amylase	3.3	62.5	96.0	100.0	Present
Without malt amylase	2.2	82.5	112.5	86.2	Absent
C With malt amylase	2.1	50.0	100.0	100.0	Absent
Without malt amylase	1.9	85.0	125.0	100.0	Absent
S With malt amylase	2.1	85.0	115.0	100.0	Absent

potatoes, 100 gm. of liver sausage, 30 gm. of butter and 250 cc. of milk. The milk was ingested during the meal. The malt extract (5.5 gm.), when used, was dissolved in the milk. The meal was ingested 12 hours after the last meal, all saliva being swallowed. Two hours later the entire gastric contents

were aspirated, the volume noted, and a sample used for pH and acidity determinations. One portion was immediately inactivated and the concentration of reducing sugar was determined immediately; and another sample was incubated for 2 hours with an excess of malt amylase to determine the per cent of starch digestion; still another portion was assayed for active malt amylase. From these data the percentage of gastric starch digestion was calculated, the total yield of reducing sugar from the meal having been previously determined.

The results are shown in table 12. It is to be noted that in four of the ten subjects (J.B., W.B., M.D. and C) the addition of malt amylase increased the gastric digestion of starch. In these subjects the pH of the gastric contents was sufficient to destroy ptyalin but not malt amylase. In the other subjects complete digestion of the starch occurred in the stomach without the addition of malt amylase. (Of course, some undigested starch entered the intestine.)

The interpretation of the foregoing results, we believe, is quite obvious. In the four of the subjects the ptyalin digestion of starch in the stomach was incomplete. An adequate amount of ptyalin was not present during the initial period of gastric digestion to effect complete gastric digestion of starch before the acidity rose to the point at which ptyalin is inactivated. In the other subjects the ptyalin was adequate to effect complete gastric digestion of starch before the acidity rose to the point of inactivation of ptyalin, and the addition of malt amylase was superfluous.

#### DISCUSSION

The observations on dogs are not considered to require discussion because the results are so clear cut and their interpretation so obvious. To our knowledge no previous work of this nature has been done on dogs. These observations on dogs form the basis of studies now under way on the effectiveness of orally administered malt amylase in the presence of pancreatic deficiency.

The observations on the human subjects may be discussed in the light of those of Bergeim ('26) and Müller ('01). Müller used rice mush as a test meal and found that from 59 to 80 % of the starch was rendered soluble. Using bread in four subjects, the figures observed by Müller varied from 50 to 77 %, the starch digestion being less when the bread was poorly masticated. Bergeim, using potatoes (six subjects) obtained figures varying from 54 to 84 % (average 76), and using bread (six subjects) obtained figures varying from 46 to 68 % (average 59). Bergeim requested his subjects not to swallow saliva prior to the meal and to masticate in a reasonably thorough manner. About half an hour after the first of the food was ingested and at 15-minute periods thereafter, samples were removed for assay. He added ferric hydrate to his meal for the same reason that we added phenol red. For cereal (farina) in our 'restricted salivary amylase control' experiments (table 10), the per cent of digestion (reducing sugars) varied from 26 to 84%, average 45. This is less than that observed by the above observers, because in these experiments we lavaged the stomach of our subjects and had them expectorate saliva after the meal to simulate a salivary deficiency. In the case of our mixed meal (table 12) in which potatoes (we used 200 gm.; Bergeim 100 gm.) served as the starch substrate, we observed from 72 to 100 % digestion of starch. These figures are higher than those of Bergeim, because protein (liver sausage and milk) was present in our meal which buffered acid and permitted the salivary amylase to act longer.

#### SUMMARY

1. When cooked starch containing malt extract was placed in the stomach of dogs with a pouch of the entire stomach and retained there and histamine injected to stimulate the secretion of the pouch, 20 % (corrected) of the starch was digested in 1 hour before the acidity rose to a point at which malt amylase is inactivated. Ninety-five per cent of the digestion occurred in the first 30 minutes.

2. A. In dogs with a complete duodenal fistula with the bile and pancreatic juice excluded, the addition of malt extract to a meal of cereal ('farina') appreciably facilitated gastric emptying because of the liquefying action of amylase. The addition of malt amylase increased the digestion of the starch from a control value of from 5 to 12 % to a value of from 30 to 65 % (average increase 370 %). About 87 % of the amylase passed into the duodenum in active form in these experiments. Variations in the per cent of starch digestion were directly correlated with the rate of evacuation and the rise in acidity of the gastric contents.

B. When a known quantity of malt amylase in water was drunk after the ingestion of a cereal meal, 75 % of the enzyme passed into the duodenum within 10 minutes the remainder becoming mixed with the gastric contents to promote the gastric digestion of starch (16 to 20 % increase). Thus, if it is desired to introduce malt amylase rapidly into the intestine in active form, this is best accomplished by taking the enzyme in water after a meal (*vide infra*).

3. A. The results obtained on dogs with an incomplete duodenal fistula and with pancreatic juice and bile excluded compared favorably with those on the complete duodenal fistula dogs, except that the percentage gastric digestion of starch was somewhat less on the average, varying from 30 to 40 %. This was due to the more rapid rise in gastric acidity in incomplete duodenal fistula dogs.

B. When a diastatically equivalent amount of human saliva was substituted for malt amylase, considerably more starch digestion resulted with malt amylase. This is due to the fact that ptyalin, which is inactivated at pH 4.5 does not act as long in the stomach as malt amylase which is inactivated at pH 2.5.

C. When the experiment cited under 2 B above was repeated on these dogs, which more closely simulate the normal, both milk and water being used as a solvent of the malt extract, it was found that with water from 69 to 71 % of the enzyme is obtained from the duodenum in 30 minutes, whereas with milk



34 to 54 % of the enzyme is obtained, because the milk does not leave the stomach as rapidly as water. The milk buffers acid and the acidity rises somewhat more slowly. However, with the administration of an excess of malt amylase little difference, if any, in the amount of gastric digestion of starch would obtain.

4. A. In experiments on human subjects performed so as to simulate a salivary deficiency, the addition of malt extract to a cereal meal definitely augmented the gastric digestion of starch in seven out of eight subjects. The variations observed depended upon the rate at which the acidity of the gastric contents rose.

B. In experiments designed to ascertain the amount of active malt amylase given with a cereal meal that passes into the intestine before the acidity rises to the lethal point of the enzyme, the minimum figure of 42 and a maximum of approximately 100 % was obtained, the average being 51 %.

C. In experiments in which salivary amylase was permitted to play its normal role and a mixed meal (mashed potatoes, milk, liver sausage and butter), containing a moderate amount of acid buffering substances was ingested after which milk with and without malt extract added was drunk, it was found that the addition of malt amylase increased the gastric digestion of starch in four of ten subjects. In six the gastric digestion of starch was completed by ptyalin and the addition of malt amylase was superfluous. Thus, in the larger percentage of normal human subjects who partake of a mixed meal containing a moderate amount of substances which buffer acid, the gastric starch digestion resulting from salivary amylase is practically complete.

5. Neither salivary nor malt amylase after being inactivated in the stomach are reactivated in the intestine. This confirms Bergeim.

## COMMENT

In a minority of normal persons the ingestion of malt amylase adds to the salivary amylase swallowed with a meal and exerts an additional influence on the gastric digestion of starch. This influence is greater in the presence of a deficiency of salivary amylase. However, in the presence of an adequate secretion of pancreatic (amylase) and intestinal amylase (the amylase of succus entericus), the addition of malt amylase to the meal may be considered as superfluous. However, in the presence of a deficiency of pancreatic amylase and of an inadequate secretion of saliva, which might be due to defective ptyalin formation or to the bolting of food in a person whose gastric acidity rises rather quickly to the lethal point of ptyalin, the addition of malt amylase to the meal is definitely indicated by our results. Actual tests on dogs and on patients suffering a deficiency of pancreatic secretion are necessary to substantiate this reasonable a priori deduction.

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# A TECHNIC FOR DETERMINING THE RATE OF ABSORPTION OF FATS<sup>1, 2</sup>

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ONE FIGURE

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## INTRODUCTION

The rate of absorption of fat may be measured directly by determining the amount of fat remaining in the alimentary tract at intervals after feeding fat, or indirectly by following the appearance of fat in the blood stream. The latter measurement is, of course, influenced by factors other than the actual absorption of fat from the tract as, for example, the rate of metabolism, deposition of fat in the tissues, and mobilization of fat from the tissues. Obviously, the direct method is preferable if one is interested primarily in absorption. In this laboratory we have adapted the technic as developed by Cori ('25) for sugars to the determination of the rate of absorption of fats. In applying this technic certain modifications had to be made which are described in detail in this report.

Two methods for determining blood fat were also tried, the chylomicron method of Gage and Fish ('24) and the haemolipokrit method of Rückert ('31). Each of these has its own intrinsic value, but neither was satisfactory for our purpose, as will be pointed out later.

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<sup>2</sup> Published with the permission of the director of the Wisconsin Agricultural Experiment Station, Madison.

## EXPERIMENTAL

*Direct method of determining fat absorption*

*Fasting.* Adult male rats 4 to 7 months of age and weighing between 200 and 400 gm. were fasted 48 hours before they were fed a fatty meal. It was found necessary to fast the animals this length of time to empty the stomach and small intestines of food previously eaten. No amount of fasting up to 1 week emptied the caecum. During the 48-hour fast distilled water only was available, and the animals lost 9.4 % of their weight.

*Feeding.* In order to feed a measured quantity of fat the animals were lightly anesthetized with ether, a mouthpiece was placed behind the incisors, and a small (no. 8) urethral catheter inserted into the stomach. By means of a 2 cc. hypodermic syringe attached to the catheter 1.5 cc. of melted fat was delivered directly into the stomach. In a similar manner 1.5 cc. of fat was delivered from the syringe directly into a beaker and weighed. The average value of several such weighings was used in all calculations as the weight of the fat fed. These weights checked to within 0.01 gm.

*Removing the contents of the alimentary tract.* When the desired time had elapsed after feeding the fat the contents of the intestinal tract were removed. The rats were decapitated, the abdominal cavity opened, and ligatures placed at the oesophageal sphincter and at the caecal-colonic juncture. The stomach, small intestine, and caecum were removed immediately from the body cavity. For convenience in handling, ligatures were also tied at the pyloric sphincter, at the ileo-caecal valve, and at the middle of the intestine. The stomach and caecum were severed from the intestine, and the intestine was cut into two parts. By means of a hypodermic syringe each section was filled with distilled water. After 10 minutes the contents of the stomach and intestine were emptied into one beaker and the contents of the caecum into another. The sections were then filled with petroleum ether to dissolve the remaining fat, and after another 10 minutes

the contents were emptied into the appropriate beakers. The stomach and caecum were cut wide open and washed thoroughly with a jet of water and petroleum ether. The intestine was stripped manually to remove the last vestige of food. The contents of the stomach and intestine and the contents of the caecum were extracted separately because the caecum often contained a viscous material that made extraction in large volume difficult. Petroleum ether was used as the fat solvent because of its insolubility in water, its good solvent properties, and its relative physiological inertness.

*Extraction of fat from the alimentary residues.* The extraction of fat from the collected washings was continued with petroleum ether. The washings were transferred to a 125 cc. separatory funnel, petroleum ether added to make the total volume approximately 25 cc., and extracted vigorously. In all, four extractions were made. The ether extracts were combined (total volume about 100 cc.) and 1 gm. of anhydrous sodium sulfate added. The extract was then filtered into a tared beaker and the sulfate washed thoroughly. After standing over night the beakers were placed in a vacuum oven at 92 to 95°C. for 3 hours. For the first  $\frac{1}{2}$  hour the pressure was held at 610 mm. Hg; it was then lowered to 85 mm. Hg and maintained at 85 mm. Hg during the remaining  $2\frac{1}{2}$  hours. After cooling in a desiccator, the beakers were weighed.

To test the accuracy of this technic, groups of rats were fed 1.5 cc. of melted fat, killed immediately, and an attempt made to recover the fat by the method described. In all cases the percentage recovery was between 95 and 100 %. For instance, ten such animals when fed a hydrogenated fat showed a mean percentage recovery of 97.5 % (range 96.3 to 100.0 %). Another group of ten rats fed corn oil showed a mean percentage recovery of 97.8 % (range 95.0 to 99.1 %). It was apparent that the method used was sufficiently accurate for biological work of the character undertaken.

A small but significant amount of fat was always found in the caecum. In most cases the mean weight of the caecal fat

increased up to 6 or 8 hours after a meal of fat and then decreased. For example, after feeding 1.3550 gm. of butter fat, the mean amounts of fat extracted from the caecal contents at 2, 4, 6, 8 and 12 hours after feeding were 0.0319, 0.0363, 0.0483, 0.0426 and 0.0312 gm., respectively. The mean amount of fat found in the caecal contents after 48 hours of fasting was 0.0086 gm.

TABLE 1

*Individual experiments showing the percentage of fat absorbed by adult male rats fed halibut liver oil*

RAT NO.	GRAMS OF HALIBUT LIVER OIL FED	ABSORPTION TIME	WEIGHT OF FAT IN CONTENTS OF STOMACH AND INTESTINES	WEIGHT OF FAT IN CAECUM CONTENTS	CORRECTED WEIGHT OF FAT RECOVERED <sup>1</sup>	TOTAL WEIGHT OF FAT ABSORBED	PERCENTAGE OF FAT ABSORBED
		<i>hours</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	
♂ 324	1.3656	2	0.8980	0.0250	0.9008	0.4648	34.0
♂ 329	1.3656	2	0.8548	0.0210	0.8536	0.5120	37.5
♂ 330	1.3656	2	0.9639	0.0166	0.9583	0.4073	29.8
♂ 349	1.3656	2	0.8228	0.0098	0.8104	0.5552	40.7
♂ 499	1.3656	2	0.8616	0.0220	0.8614	0.5042	36.9
♂ 500	1.3656	2	0.7824	0.0116	0.7718	0.5938	43.4
♂ 501	1.3656	2	0.9754	0.0140	0.9672	0.3984	29.1
♂ 507	1.3656	2	0.8107	0.0187	0.8072	0.5584	40.8
♂ 323	1.3656	2	0.6558	0.0092	0.6428	0.7228	52.9
♂ 508	1.3656	2	0.7193	0.0147	0.7118	0.6538	47.8
♂ 509	1.3656	2	0.8280	0.0186	0.8244	0.5412	39.6
Mean							39.4±1.6

<sup>1</sup> The values in this column are the sum of the weights given in the two preceding columns minus the correction factor, 0.0222 gm.

*Correction factor.* In calculating absorption values it was essential to know how much fat was present in the intestinal tract after the 48-hour fast. A group of seven rats was, therefore, fasted, killed, and the fat extracted by the method described above. The mean weight of the fat obtained by this procedure was 0.0222 gm. This correction was applied in each case before the percentage absorption was calculated.

*Results.* In table 1 are given the complete data for a group of eleven rats fed 1.5 cc. of halibut liver oil and killed 2 hours later. At first glance it might appear that the range, 29 to 53,

is large, but throughout all of our experiments on absorption similar variations were found. A range greater than this was almost never encountered, and usually the extreme values fell within the range of  $M \pm 10$  to 12. The probable errors as shown in table 2 were typical of all experiments. Apparently these variations are an expression of the innate differences in the ability of the animals to absorb fat.

*The effect of the anesthetic.* In order to determine whether or not etherization had any effect upon the rate of fat absorption, groups of rats were kept under ether for 30 minutes, 10 minutes, 5 minutes, and 1 minute before feeding, and the percentage of fat absorbed in 4 hours determined. A fourth group of rats was not anesthetized but was offered 1.5 cc.

TABLE 2

*The effect of ether upon the percentage of fat absorbed in 4 hours by adult male rats fed 1.5 cc. of corn oil*

TREATMENT	NUMBER OF RATS	MEAN PERCENTAGE ABSORPTION
No ether	8	61 $\pm$ 0.9
Etherized 1 minute	10	58 $\pm$ 0.9
Etherized 5 minutes	6	57 $\pm$ 1.9
Etherized 10 minutes	6	52 $\pm$ 1.1
Etherized 30 minutes	6	37 $\pm$ 2.7

of fat in a small dish. If the animal did not consume the fat in 10 minutes, as determined by weighing the dish before and after feeding, it was not included. Forced feeding without etherization was tried, but we came to the conclusion that light anesthesia was much less disturbing to the animal than the excitement caused by forced feeding.

It is apparent from the results (table 2) that prolonged etherization had an effect upon absorption. However, the shorter periods of anesthetization, 1 and 5 minutes, had no significant effect. (Statistical analyses have shown that for a group of ten animals a mean difference of around 10 % is necessary to give significance.)

Our attempts to secure voluntary consumption of small amounts of fat in a short enough period of time to avoid the



introduction of a serious error were found highly impractical. It was decided, therefore, to anesthetize the rats the shortest possible time and to use a standard procedure for all the animals. The method adopted was as follows:

Three cubic centimeters of anesthesia ether were poured onto one-half of a paper towel which had been placed in the bottom of an open ether can of 4-liter capacity. The can was covered immediately with a heavy glass cover and the ether allowed to vaporize for 3 minutes at room temperature. The animal was then introduced and removed just as soon as it had relaxed. The fat was fed at once. Invariably the animal recovered consciousness and walked around the cage with perfect equilibrium 1 to 2 minutes after the fat was administered.

*The amount of fat fed.* Gross trial experiments showed that as much as 1.5 cc. of fat could be fed successfully to our adult male rats and still leave sufficient residue of fat in the intestine after 6 hours for satisfactory analysis.

In view of the conflicting statements (Cori, Cori and Goltz, '29; Cori, Villiaume and Cori, '30; Cori, '30; MacKay and Bergman, '33; Magee and Reid, '31; Pierce, Osgood and Polansky, '29; and Wilson and Lewis, '29) concerning the effect of the amount of sugar fed upon its rate of absorption, the amount of fat fed to our rats was increased and the results observed. Previously 1.5 cc. (1.3434 gm.) of a commercially hydrogenated vegetable oil had been fed to groups of ten rats each and the percentage of fat absorbed at 2, 4, 6, 8 and 10 hours determined. We now fed twice as much, namely, 2.6868 gm. of the same fat and determined the absorption. The results are shown in table 3. Greater irregularities were encountered when 3 cc. instead of 1.5 cc. of fat were fed. As a matter of fact, a number of the animals on the larger amount had diarrhea and had to be discarded. Apparently 3 cc. of fat, when administered according to our technic, was more than our rats could digest. Consequently 1.5 cc. of fat was fed in all subsequent experiments.

*The effect of age.* To determine if age is a factor influencing the rate of absorption of fat, nine rats that were 10 months of age were fed in comparison with others. The mean percentage of fat absorbed by these animals was 3 % lower than the general average of our rats 4 to 7 months old—a difference which is not significant. Again, a group of five young rats 40 to 50 days of age were tested. In 4 hours these rats absorbed  $56 \pm 2.4$  % of the fat fed, whereas animals that were 4 to 7 months of age absorbed  $54 \pm 1.6$  % of the same fat. Obviously, age, within the limits of our experiment, namely 4 to 7 months, did not influence the rate of absorption to any great extent.

TABLE 3

*The percentages of fat absorbed by groups of adult male rats fed 1.5 cc. or 3 cc. of fat*

TIME	FED 1.5 CC. OF FAT		FED 3 CC. OF FAT	
	Mean number of milligrams absorbed	Mean percentage absorbed	Mean number of milligrams absorbed	Mean percentage absorbed
<i>hours</i>				
2	$357.6 \pm 20.6$	$26.6 \pm 1.5$	$509.7 \pm 77.8$	$19.1 \pm 0.9$
4	$723.2 \pm 21.2$	$53.8 \pm 1.6$	$780.5 \pm 34.1$	$29.0 \pm 1.2$
6	$918.8 \pm 24.2$	$68.5 \pm 1.7$	$1183.5 \pm 39.1$	$44.0 \pm 1.3$
8	$1155.9 \pm 16.6$	$86.0 \pm 1.3$	$1383.4 \pm 45.9$	$51.4 \pm 1.7$
10	.....	.....	$2143.9 \pm 38.7$	$79.7 \pm 0.5^1$
12	$1325.3 \pm 3.5$	$98.6 \pm 0.3$	$2463.1 \pm 14.7$	$91.6 \pm 0.6$

<sup>1</sup> This group contained only seven animals.

*Sex as a factor in absorption.* To determine the influence of sex a comparison of the absorption of adult male and female rats was made. The females lost on the average 9.9 % and the males 9.4 % of their weight during the 48-hour fast. The mean differences between the percentages of fat absorbed by male and female rats (table 4) are not statistically significant.

*The effect of pregnancy.* To test the effect of pregnancy on the absorption of fat five females between the second and third week of pregnancy were fed 1.5 cc. of a hydrogenated fat and killed after 4 hours. The results were no different

from those obtained from a group of eleven male rats similarly treated; the females absorbed a mean of  $50.3 \pm 3.7\%$  of the fat fed; the males,  $52.8 \pm 2.4\%$ .

*Seasonal variations.* For the most part our experiments were done during the spring and summer of 1933. In the fall of 1934 after a particularly hot summer a large number of female rats in the stock colony were not breeding normally.

TABLE 4

*The percentage of fat absorbed by male and female rats at 2, 4, 6, 8 and 12 hours after feeding 1.5 cc. of fat*

	NUMBER OF RATS	MEAN WEIGHT	MEAN PERCENTAGE FAT ABSORBED
		gm.	
Absorption time			
2 hours			
Male	25	286	$26.3 \pm 0.57$
Female	15	225	$23.9 \pm 0.65$
Absorption time			
6 hours			
Male	27	273	$64.2 \pm 0.94$
Female	19	215	$63.3 \pm 1.25$
Absorption time			
8 hours			
Male	7	283	$85.7 \pm 1.63$
Female	2	216	83.0 ...
Absorption time			
12 hours			
Male	10	267	$99.4 \pm 0.15$
Female	6	218	$99.2 \pm 0.30$

Rather than discard them outright, an attempt was made to use them in fat-absorption tests. At the time, when controls were run on the base fat, a hydrogenated vegetable oil, a mean of  $45.8 \pm 1.6\%$  of the fat was absorbed by a group of ten female rats, whereas the mean obtained previously for this fat was  $53.8 \pm 1.6\%$ . The experiment was repeated immediately using six male rats. The mean percentage absorption was  $52.3 \pm 0.9\%$ , a figure comparable to the 1933 value. The absorption of corn oil was checked also. The

1933 value for male rats fed corn oil was  $58.3 \pm 0.9 \%$ ; the 1934 value,  $61.0 \pm 1.5 \%$ . Apparently the physical condition of the female rats that we were attempting to use was affecting their ability to absorb fat. We, accordingly, found it necessary to confine our comparisons to experiments carried out within periods of limited time and on animals in a similar physiological condition.

*Correlation coefficients between body weight, body surface, length of intestines, and the grams of fat absorbed.* Cori ('25) assumed that the amount of sugar absorbed by a rat is proportional to body weight. He, therefore, expressed his results as a coefficient calculated by the formula  $\frac{A \times 100}{W \times T}$ , wherein A represents the milligrams absorbed, W the weight of the animal after fasting, and T the time of absorption in hours. This coefficient of absorption represents the milligrams absorbed per 100 gm. of body weight per hour of time. Pierce, Osgood and Polansky ('29) and MacKay and Bergman ('33) state that the rate of absorption of glucose appears to be more closely related to body surface than to body weight. They substitute body surface for body weight in the formula above and express the coefficient of absorption as the milligrams of glucose absorbed per square centimeter of body surface per hour. Cori used young and growing animals, whereas we used adults. In order to determine to what extent the amount of fat absorbed by our animals was dependent upon body weight, body surface, or intestinal surface, correlation coefficients were calculated between these variables. The formula of Carman and Mitchell ('26),  $S = 11.36 \text{ wt.}^{2/3}$ , was used in calculating body surface. The intestinal surface was assumed to be proportional to the length of the intestine. It was found that the measured length of the removed intestine varied greatly with the method of removing it from the body. However, if the mesentery was pulled off as the organ was removed and the intestines stretched carefully, more drastic treatments, even stripping manually to remove the contents, did not alter the length more than a few centimeters.

The correlation coefficients and their probable errors at the different absorption periods are shown in table 5. The correlations between the grams of fat absorbed and the length of the intestines show that there is no striking relation between these factors. The mean length of the intestine for twenty-one female rats upon which this measurement was taken was 93.9 cm. with a range of 65 to 108 cm. A similar measurement on fifty male rats was 93.3 cm. with a range of 69 to 115 cm. From the physiological point of view it is quite possible that the conditions of our experiment in no way taxed the ability of the intestinal surface to absorb fat into the blood stream. Moreover, the intestinal surface is only one of the possible limiting factors in the absorption

TABLE 5

*Correlation coefficients between body weight, length of intestine, body surface, and the grams of fat absorbed*

ABSORPTION TIME IN HOURS	NUMBER OF OBSERVA- TIONS	CORRELATION BETWEEN BODY WEIGHT AND GRAMS OF FAT ABSORBED	CORRELATION BETWEEN LENGTH OF INTESTINE AND GRAMS OF FAT ABSORBED	CORRELATION BETWEEN BODY SURFACE AND GRAMS OF FAT ABSORBED
2	15	$-0.32 \pm 0.16$	$-0.36 \pm 0.15$	$-0.32 \pm 0.16$
4	41	$+0.27 \pm 0.10$	$+0.17 \pm 0.07$	$+0.27 \pm 0.10$
6	41	$+0.38 \pm 0.09$	$+0.32 \pm 0.09$	$+0.27 \pm 0.10$
8	38	$+0.36 \pm 0.09$	$+0.25 \pm 0.10$	$+0.41 \pm 0.09$

of fat; it may under proper circumstances play a role of secondary importance to the secretion of digestive juices, the rapidity and ease of emulsification, the concentration of fat in the blood stream, and many others.

Of the correlation coefficients given in the table only one of them is three times as large as its standard deviation, namely the correlation between body surface and the grams of fat absorbed at 8 hours. This correlation is not supported by similar correlations at the other absorption periods, so no especial significance can be attributed to it. Since no very definite or striking correlations exist between the measured variables, there is no logic in relating absorption to any one of them. Our results, therefore, are expressed simply as percentage of the fat absorbed.

*The determination of blood fat*

*Chylomicron method.* Various attempts were made to increase the accuracy of the chylomicron method, but no measurable success was attained. The technic finally adopted was essentially that proposed by Gage and Fish ('24) in their original article. Other micro methods of determining blood fat are reviewed by Man and Gildea ('32).

The rats were fasted 24 hours and then fed 0.5 cc. of fat by stomach tube. Five-tenths cubic centimeter was fed instead of 1.5 cc. to keep the number of chylomicrons appearing in the blood from becoming too numerous to count. A small drop of blood from the end of a rat's tail was touched to a coverslip (no. I, 1 sq.cm.) which was immediately placed in contact with a glass slide and weighted with a 500-gm. weight for 10 seconds. The size of the drop taken was such that the blood film covered about three-fourths of the area of the coverslip. Duplicate samples were mounted on one slide and the edges sealed with mineral oil. Counting was usually completed within an hour after the collection of the sample. A Leitz microscope equipped with a dark field, a 1/12 oil immersion objective, a 10 × ocular, and an ocular net 5 mm. × 5 mm., graduated in 0.5 mm., were used. A field was considered suitable for counting when about one-fourth of the area of the net was not occupied with blood cells and was without fibrin clots and/or oxyhemoglobin crystals. The number of moving particles in each of four  $\frac{1}{4}$  sq.mm. was counted in a given field. Ten fields were counted and an average value calculated for the number of chylomicrons in 1 sq.mm.

Obviously, there are many errors in this technic. When the number of chylomicrons is large, the accuracy of counting them with their brownian movement is greatly diminished. Consequently, we attempted to dilute the blood. By means of suitable pipettes dilutions of 1:200, 1:400, 1:800 and 1:1600 of blood in 1 % acetic acid solution were made. A 1 % acetic acid solution was used in preference to a physiological saline because it laked the red blood cells and thus

helped to clear the field. When counts were made, the expected relation between the different dilutions was by no means always obtained.

Both clotted blood and oxalated blood were centrifuged and counts made on the serum. The interference of the blood cells was thereby eliminated, but when the cells themselves were suspended in saline solution and viewed under the microscope, the mount was teeming with chylomicrons.

Attention was then directed toward the use of a counting chamber in order to insure a definite depth of solution. The Petroff-Hauser counting chamber was found to be as satisfactory as any, but by focusing up and down at different levels we were able to bring almost any number of chylomicrons into view.

To test the effect of fasting the animal upon the number of chylomicrons in the blood, three fasting periods were used—16, 24 and 48 hours. No differences in the response of the animals could be detected.

In another experiment rats were fasted 24 hours, anesthetized, and kept under the influence of ether for 15 minutes before they were fed 0.5 cc. of fat. No effects due to the prolonged etherization could be demonstrated.

Rats were exercised either 15 or 30 minutes before feeding fat by allowing them to swim in a tank of water at 35°C. Other animals were massaged gently for similar periods before feeding. Although the number of animals was too few to draw final conclusions, it appeared that both exercise and massage caused a slight increase in the number of chylomicrons.

The effect of temperature was studied by counting the chylomicrons in the blood of rats kept at 14°C. and at 48°C. during the fasting period. After feeding 0.5 cc. of fat, counts were made in the usual manner. It appeared that the low temperature did not affect the chylomicron response, but the higher temperature seemed to reduce it. No differences were noted at the end of the fasting period before fat was fed.

It was thought that if a fat-free diet could be substituted for the fasting period, the response of the rats would more nearly approach a normal physiological response. The number of chylomicrons in the blood was as low when a fat-free diet had been fed for 48 to 72 hours as when the animal was fasted. When fat was fed to the animals on a fat-free diet, the peak of the curve was lower and tended to occur later than when the animals were fasted.

The number of chylomicrons appearing in the blood of rats fed a fat-free diet for 72 hours and then fed 0.5 cc. of various fats was determined. Typical curves for two of the fats tested, namely butter oil and halibut liver oil, are given in figure 1. There was a definite tendency for the curves to be of the same general outline when a given rat was fed the same fat at different times. Variations in successive responses of one rat were not as great as variations between the responses of different rats receiving the same fat. However, variations were so great that we were unable to demonstrate any differences in the rate of absorption. We concluded, therefore, that the chylomicron method makes an interesting demonstration of an increase in blood fat following the ingestion of fat, but that for our purpose it had little or no quantitative value.

*Haemolipokrit method.* The haemolipokrit method of Rückert ('31) was found to be much more satisfactory as a quantitative measure than the chylomicron method. The technic used was essentially that described in detail by Herrmann, Ames and Tapke ('34).

The animals were fasted 48 hours, fed 1.5 cc. of fat, and at the proper intervals thereafter anesthetized with ether and 3 cc. of blood drawn by heart puncture. The blood was allowed to stand 15 minutes at 37°C. to facilitate clotting before it was centrifuged. The serum obtained was either clear or slightly milky due to the presence of fat particles.

Duplicate analyses on the same sample of serum gave concordant results, but the variations from rat to rat were fairly large. For instance, one group of ten rats bled 4 hours after



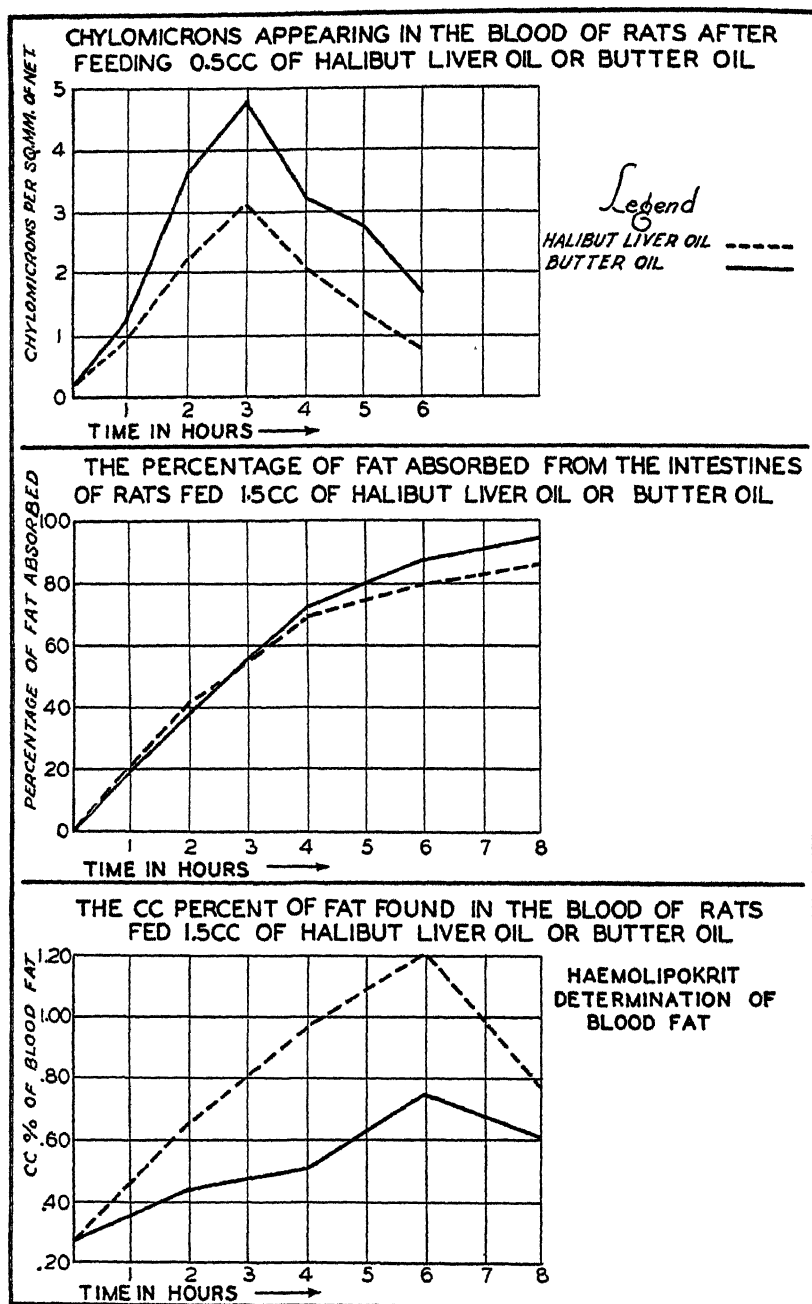


Fig. 1 Each point on the curves is the mean of ten or more individual values.

receiving halibut liver oil gave values for blood fat ranging from 0.62 cc. % to 1.22 cc. % with a mean value of  $0.97 \pm 0.04$  cc. %. Whereas these variations are greater than those obtained when the rate of absorption of fat from the intestine was measured directly, they are perhaps to be expected, since one is measuring absorption from the intestine as influenced by the factors of metabolism.

In figure 1 are given the blood fat curves for groups of rats fed halibut liver oil or butter oil.<sup>3</sup> It will be noted that the chylomicron and haemolipokrit curves for blood fat do not substantiate each other. The haemolipokrit values only can be considered significant and indicate a definite difference in the rate of appearance of fat in the blood when butter oil and halibut liver oil are fed. Determinations of the rate of absorption of these two fats show that they disappear from the alimentary tract at approximately the same rate. According to Hughes and Wimmer ('35) the shorter chain, more soluble fatty acids are absorbed directly into the portal circulation and are not resynthesized into fat as they pass through the intestinal wall. If this is the case, it might account for the smaller amounts of butter oil found in the blood. It is also possible that the shorter chain fatty acids are metabolized or stored more rapidly in the body than the longer chain acids.

Since we were interested in determining the rate of absorption of fat from the tract rather than in studying blood fat and since the results on blood fat did not parallel the data on absorption, we limited our work to the direct method of analyzing the intestinal residues.

<sup>3</sup>Preparation of butter oil. Butter was melted and the butter fat separated by decantation; 240 gm. of the butter fat was dissolved in 1500 cc. of absolute alcohol at 45°C. and then cooled to 1°C. The mixture was filtered and the alcohol removed from the filtrate by distillation in a partial vacuum with nitrogen at 40 to 45°C.; 33 gm. of butter oil were obtained.

## SUMMARY

A method for determining the comparative rate of absorption of fat from the alimentary tract has been described in detail. The method involves the feeding of a definite quantity of fat and subsequent analysis of the intestinal residues. Concomitant use of the chylomicron method and the haemolipokrit method for determining blood fat did not give comparable results. The former was found to have little or no quantitative value. The haemolipokrit method gave concordant results on duplicate samples of serum but was not found satisfactory for studying the rate of absorption of fats because different fats appeared in the blood at rates quite different from those with which they disappeared from the alimentary tract.

No definite or striking correlations between the grams of fat absorbed and the variables—body weight, body surface, or length of intestines—were found.

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# THE COMPARATIVE RATE OF ABSORPTION OF DIFFERENT FATS<sup>1, 2</sup>

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## INTRODUCTION

The literature dealing with the absorption of fats has been adequately reviewed by Verzar ('33). Of the various investigators, Holmes and co-workers (Deuel and Holmes, '22; Holmes, '18 a, '18 b, '19 a, '19 b; Holmes and Deuel, '20, '21; and Langworthy and Holmes, '15, '17 a, '17 b) have specialized in studies of absorbability of fats. So far, however, little work has been done on the comparative rate of absorption of different fats. It is this phase of the physiology of fats with which our present experimental work deals. We found that certain fats are more rapidly absorbed than others, but whether or not rapidity of absorption is physiologically advantageous is beyond the scope of this paper. We merely wish to present the data at hand.

## EXPERIMENTAL

The technic used in determining the rate of absorption of fats has already been described by Irwin, Steenbock and Templin ('36). Briefly, it consisted of a modified Cori ('25) method in which adult male rats fasted for 48 hours were lightly anesthetized with ether and fed 1.5 cc. of melted fat

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<sup>2</sup> Published with the permission of the director of the Wisconsin Agricultural Experiment Station, Madison.

by stomach tube. At intervals thereafter the animals were killed and the contents of the alimentary tract analyzed for fat.

The fats studied were lard, corn oil, two hydrogenated vegetable oil shortenings designated as A and B, respectively, butter fat, butter oil, halibut liver oil, and cod liver oil. These were considered as representative of a wide variety of fats. They were fed to groups of ten or more rats and the percentage of fat absorbed at 2, 4, 6, 8 and 12 hours determined. All the fats except the butter fat and the butter oil were commercial products.<sup>3</sup> The butter fat used was prepared from butter as churned from sweet cream. No salt or coloring matter was added. The butter was melted and the butter fat separated from the water and casein by decantation. The butter oil was prepared by dissolving butter fat in absolute alcohol at 45°C. and then cooling the mixture to 1°C. The mixture was filtered and the alcohol removed from the filtrate by distillation in a partial vacuum with nitrogen at 40 to 45°C. The oil as obtained from 240 gm. of butter fat dissolved in 1500 cc. of alcohol weighed 33 gm. All of the solid fats were melted and fed at temperatures a few degrees above their melting points.

The mean percentages of fat absorbed together with their probable errors for the different absorption periods are given in table 1. The results of 342 individual experiments are represented by these data. It will be seen that the percentage of fat absorbed at the different absorption periods was practically the same when lard, corn oil, or the hydrogenated shortenings were fed. Butter oil, halibut liver oil, and cod liver oil were absorbed at similar rates but were definitely more rapidly absorbed than the group just mentioned. The rate of absorption of butter fat falls somewhere between these two groups.

<sup>3</sup> The fats and oils used were those available commercially. Hydrogenated shortening A was manufactured by hydrogenating cottonseed oil to an iodine number of approximately 70. The saponification number was 196.0. For shortening B corresponding data were not available.

Corn oil was chosen as the fat with which to compare butter fat, butter oil, halibut liver oil, and cod liver oil in calculating the statistical significance of the differences in the percentages of fat absorbed because the percentage of corn oil absorbed at the different absorption periods was

TABLE 1

*The mean percentages of fat absorbed by rats fed 1.5 cc. of fat<sup>1</sup>*

KIND OF FAT FED	ABSORPTION TIME				
	2 hours	4 hours	6 hours	8 hours	12 hours
Lard	24.1±0.8	57.0±1.5	67.5±1.5	92.3±0.9	97.8±0.4
Corn oil	28.9±0.8	58.3±0.9	71.4±2.1	94.4±0.7	97.9±0.3
Shortening B	27.1±1.8	52.8±2.4	71.1±1.5	85.6±1.2	99.6±0.1
Shortening A	26.6±1.5	53.8±1.6	68.5±1.7	86.0±1.3	98.6±0.3
Butter fat	36.2±1.6	60.3±1.2	77.2±2.0	91.2±1.1	97.4±0.4
Butter oil	37.4±2.3	71.0±1.2	86.4±1.7	95.6±1.0	.....
Halibut liver oil	39.4±1.6	70.2±2.0	78.1±1.3	85.4±0.9	.....
Cod liver oil	40.8±1.4	67.7±1.9	79.7±1.5	89.2±0.7	98.2±0.4

<sup>1</sup> All of the animals were adult male rats. Each figure is the mean of ten or more individual experiments except in the case of the 12-hour group fed cod liver oil, which figure is a mean of only five experiments.

TABLE 2

*Mean differences, standard deviations of the mean differences, and  $\frac{MD}{\sigma MD}$  for butter fat, butter oil, halibut liver oil, and cod liver oil compared with the base fat, corn oil*

	2 HOURS			4 HOURS			6 HOURS		
	MD	$\sigma MD$	$\frac{MD}{\sigma MD}$	MD	$\sigma MD$	$\frac{MD}{\sigma MD}$	MD	$\sigma MD$	$\frac{MD}{\sigma MD}$
Butter fat and corn oil	%			%			%		
Butter oil and corn oil	7.2	2.7	2.7	2.0	1.9	1.0	5.8	4.3	1.3
Halibut liver oil and corn oil	8.5	3.6	2.4	12.7	2.2	5.7	15.0	4.0	3.7
Cod liver oil and corn oil	10.5	2.6	4.0	11.9	3.3	3.6	6.7	3.6	1.8
	11.9	2.4	4.9	9.4	3.1	3.0	8.3	3.8	2.2

as high as lard or the hydrogenated fats. If the differences between butter oil, halibut liver oil, or cod liver oil and corn oil are significant, similar differences between these three fats and lard or the hydrogenated fats are also significant. The mean differences together with their standard deviations and the quotients  $\frac{MD}{\sigma MD}$  at 2, 4 and 6 hours are given in table 2.



At 8 and 12 hours so much of the fat had been absorbed that the differences were no longer accurately representative of the rate of absorption. Therefore, these differences were not included in the table. Parenthetically, it may be stated that for biological work of this nature a mean difference that is two to three times as large as its standard deviation is usually considered statistically significant. It is evident that there is a real difference in the rates of absorption of butter oil as compared with corn oil (table 2). There is likewise a difference between the rates of absorption of halibut liver oil or cod liver oil and corn oil. The differences in favor of butter fat are suggestive but are not statistically significant.

Examination of table 1 will reveal that butter oil, halibut liver oil, and cod liver oil were absorbed at approximately the same rate for the first 4 hours, but at 6 and 8 hours halibut liver oil and cod liver oil lagged behind. Since it has been shown that certain hydrocarbons and higher alcohols, especially cholesterol, are not completely absorbed (Channon and Collinson, '28; Channon and Devine, '34; and Mahdi and Channon, '33), it was thought that these differences might be due to the presence of a larger residue of unsaponifiable matter from cod liver oil and halibut liver oil. Saponification of butter oil and halibut liver oil showed that butter oil contained 1.5 % of unsaponifiable matter, whereas halibut liver oil contained 13 %. To test out this point, 1.5 cc. of these two fats were fed to groups of six male rats each. After a 6-hour absorption period the animals were killed and the percentage of unsaponifiable matter in the pooled residues from the intestines of each group determined. The residue from the animals fed butter oil contained 5.3 % unsaponifiable matter, whereas those fed halibut liver oil contained 17.4 %. Apparently the unsaponifiable fraction of the oils was more slowly absorbed than the glycerides.

To test this point further unsaponifiable matter prepared from halibut liver oil was added to butter oil to make it contain 12.4 % of the same. The mixture was then fed to groups

of six animals each which were killed at 4, 6 and 8 hours, respectively. The percentage of butter oil absorbed under these conditions was 60 % at 4 hours, 69 % at 6 hours, and 78 % at 8 hours. This makes it seem probable that the larger amount of unsaponifiable matter present in halibut liver oil and in cod liver oil accounted for at least part of the lag in the absorption of these oils in comparison with butter oil.

In order to follow more closely the changes that take place in a fat as digestion proceeds, the intestinal residues from each group of rats fed cod liver oil were analyzed for unsaponifiable matter, acid number, and iodine number for various absorption periods (table 3). The data presented

TABLE 3

*The percentage of unsaponifiable matter and the acid value expressed as percentage of oleic acid in the residues extracted from the digestive tract 2, 4, 6 and 8 hours after feeding cod liver oil*

	ORIGINAL COD LIVER OIL	INTESTINAL RESIDUES AFTER FEEDING COD LIVER OIL			
		2 hours	4 hours	6 hours	8 hours
Percentage unsaponifiable matter	2.6	3.2	7.9	6.0	8.4
Acid value as percentage oleic acid	0.7	11.9	17.4	15.6	16.1
Iodine number—Hanus	154.0	...	150.0	...	...

confirm those obtained with halibut liver oil, namely that the unsaponifiable matter was absorbed less readily than the fat itself. It is apparent also that the hydrolysis of fat into fatty acids and glycerol proceeded more rapidly than the absorption of these compounds until such time as a balance between the rate of hydrolysis and absorption was established. The change in iodine value was insignificant.

At the 2-hour absorption period butter fat was more rapidly absorbed than corn oil, but at the 4- and 6-hour periods similar differences were not apparent (table 1). Since butter fat contains a larger percentage of short chain fatty acids than corn oil and since these acids are somewhat water-soluble, it is possible that they were absorbed more rapidly than the long chain fatty acids, and, therefore, a

difference occurred at 2 hours which did not persist throughout the absorption period. To test this possibility corn oil and butter fat were fed again to groups of thirteen rats each and the percentage of fat absorbed after 2 hours determined a second time. The residues from the gastro-intestinal tract were pooled and saponification and Reichert-Meissl numbers determined. The data obtained previously for the percentage absorption of these two fats (table 1) were confirmed by this experiment, butter fat being 36 % absorbed and corn oil 32 % absorbed. Analysis of the residual butter fat from the tract gave a saponification number of 224 and a Reichert-Meissl number of 20.13, whereas the original butter fat gave values of 230 and 27.17, respectively. Although these differences are not large, they indicate that the higher percentage of short chain fatty acids is a possible explanation of the more rapid rate of absorption of butter fat, which occurs at the 2-hour absorption period only.

When 1.5 cc. of fat was fed to the rats, the 4-hour absorption period appeared to be the one most suitable for comparing the rates of absorption. At this time digestion is well under way, the after effects of administration of the fat—if any—have undoubtedly been dissipated, and the diminished concentration of the fat is probably not yet a serious factor. Accordingly, we used the 4-hour absorption period for many of the fats studied. The results are shown in table 4. Whether or not another sample of any one of these fats would have shown a similar absorption rate cannot be stated, as we used only one sample. With individual variations of the magnitude encountered a mean difference of approximately 10 % is necessary for statistical significance. It should be borne in mind, therefore, that many of these fats (table 4) do not differ greatly in their absorption rates. There is, however, a very decided difference in the rate of absorption of oleo stock (36 %) and olive oil (63 %).

It is well known that fatty livers can be produced by feeding diets containing a relatively high percentage of fat. We became interested in determining whether or not there is any

difference in the production of fatty livers by different fats and whether such differences, if they occurred, could be associated with absorption rates. Seven groups of six rats each (male rats weighing 45 to 65 gm.) were fed a diet composed of crude casein 18, brewers' yeast 8, salts (Wesson, '32) 5, cooked starch 39, and fat 30. Vitamins A and D were supplied by 1 drop of halibut liver oil fed to each rat each week. The fats used were lard, butter fat, oleo stock, shortening A, coconut oil, olive oil, and a mixture of cod liver oil and lard, one to two.

TABLE 4

*Mean percentage of fat absorbed together with the probable errors, at a 4-hour absorption period*

KIND OF FAT FED	NUMBER OF RATS	PERCENTAGE OF FAT ABSORBED
Raw linseed oil	9	67.0±0.9
Olive oil	10	63.4±1.8
Whale oil	9	62.1±1.3
Soya bean oil	10	58.5±1.4
Peanut oil	9	58.3±1.7
Rancid lard	10	53.8±1.6
Cottonseed salad oil	10	53.7±1.6
Coco butter	9	47.9±1.1
Coconut oil	9	47.4±1.6
Palm oil	10	37.4±1.5
Oleo stock	10	35.8±1.0

The animals were given these diets for a period of 3 weeks. They were then killed and the livers analyzed for fat. The mean percentages of fat expressed as linoleic acid found in the livers of the several groups were as follows: lard  $15.0 \pm 0.7$ , butter fat  $14.7 \pm 0.4$ , oleo stock  $16.1 \pm 0.3$ , shortening A  $14.5 \pm 0.6$ , coconut oil  $19.2 \pm 0.5$ , olive oil  $16.8 \pm 0.5$ , and the mixture of cod liver oil and lard  $12.4 \pm 0.8$ .

None of these differences is very marked. Possibly the lower fat content of the livers of rats fed cod liver oil can be attributed to the larger amount of nitrogenous constituents in this oil. However, within the scope of this experiment no very striking differences occurred.

## SUMMARY

The percentage of fat absorbed from the alimentary canal of rats was determined at 2, 4, 6, 8 and 12 hours after feeding definite quantities of fat. It was found that: 1) partially hydrogenated vegetable oils, as sold commercially for home and bakers' use, were absorbed as rapidly as lard or corn oil, and 2) that butter oil, halibut liver oil, and cod liver oil were absorbed uniformly at a more rapid rate than lard, corn oil, or the partially hydrogenated fats.

Eleven other samples of fat tested after a 4-hour absorption period could be arranged in the following descending order of their percentage absorption: linseed oil, olive oil, whale oil, soya bean oil, peanut oil, rancid lard, cottonseed oil, cocobutter, coconut oil, palm oil, and oleo stock. Although the difference between any of these fats and the one immediately preceding or following in the list may not be significant, certainly there were very real differences in the absorption rates of those widely separated.

Besides differences in the character of the fats themselves, it appears that the quantity of unsaponifiable matter in a crude fat may influence the rate of absorption of the total.

No very striking differences occurred in the production of fatty livers when diets containing 30 % of lard, butter fat, oleo stock, partially hydrogenated vegetable fat, coconut oil, or olive oil were fed to young, growing rats.

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## THE SPECIFIC DYNAMIC ACTION OF GLYCINE INTRAVENOUSLY ADMINISTERED TO NEPHRECTOMIZED DOGS

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In a previous publication (Eaton, Cordill and Gouaux, '35), we have shown that the work of the kidney, due to the excretion of urea, is not a factor in the specific dynamic action of proteins. In view of the calculations of Borsook and Winegarden ('31 a, '31 b, '31 c) we have performed further experiments to determine whether or not the kidney is responsible for any measurable part of the specific dynamic action of glycine. There is no deamination of glycine taking place in hepatectomized dogs and no specific dynamic action, as shown by the experiments of Wilhelmj, Bollman and Mann ('28). Hence, if the kidney should be responsible for any of the increased heat production, it would have to be by virtue of its action after deamination.

So far as we are aware, only two papers dealing with this subject have appeared. Strieck ('28) studied the increase in heat production after a meal of meat both before and after nephrectomy. Most of his experiments were done so long after the operation that one might raise the objection that the animals were very uremic. The amount of meat fed was usually different from that given before nephrectomy. Dock ('31) investigated the oxygen consumption of rats fed on high and low protein diets, both before and after nephrectomy. Although rats of both groups showed a decreased



heat production after nephrectomy, there was no significant difference between the two groups.

Our experiments were planned so that the specific dynamic action of intravenously injected glycine could be studied as soon after nephrectomy as possible, a level of basal metabolism having been established after the operation.

#### METHODS

Female dogs fasted for 24 hours previous to the experiment were used. They ranged in weight from 5.2 to 13.2 kg. In most cases, one blood sample was taken before administration of sodium amytal, which was given intraperitoneally, 60 mg. per kilogram of body weight. Additional doses of sodium amytal (50 to 100 mg. per dog) were sometimes given during the day in order to preserve a uniform degree of anesthesia. Two more blood samples were taken at approximately  $1\frac{1}{2}$  and 3 hours, respectively, after the administration of the sodium amytal.

As soon as the animals were well asleep, a catheter was inserted and the bladder thoroughly washed. A urine sample was collected for approximately 2 to 3 hours. A tracheal cannula was inserted and connected to a Tissot spirometer for collection of expired air. Three determinations of the basal metabolism were then made.

Six dogs were used to investigate the specific dynamic action of glycine under sodium amytal anesthesia. Ten grams of glycine were dissolved in 100 cc. of 0.9% sodium chloride and injected at a constant rate into the saphenous vein during a period of approximately 20 minutes. The solution was maintained at body temperature and the last portion washed in by means of 15 cc. of 0.9% sodium chloride.

Venous blood samples were taken hourly thereafter, and the urine for the period collected for analysis. Hourly basal metabolic determinations were made as nearly consecutively as possible, during the next 5 hours.

In the nephrectomized animals, both kidneys were removed through an anterior midline incision with as little trauma as possible. The time required varied from 4 to 15 minutes.

Three further determinations of basal metabolism were made within the next 2 hours and at this point another blood sample was taken. Then, 7.88 gm. of glycine were given as described above. It had been found by the Sorensen titration method that the average excretion of glycine in the urine of the six dogs, which were not nephrectomized, amounted to 2.12 gm. Therefore, this amount was deducted.

Three nephrectomized dogs were used as controls and given 115 cc. of 0.9% sodium chloride in the same way as the glycine solution had been administered.

Expired air was analyzed by means of an accurate Haldane apparatus and the results carefully checked. Heat production was calculated on the basis of the total R.Q. since no accurate means of determining the protein metabolism of the nephrectomized dogs is possible. Blood and urine urea nitrogen determinations were made by the urease aeration method of Meyers ('24). Urease was prepared by the method of Koch ('26).

TABLE 1  
*S.D.A. of glycine (controls). Ten grams glycine*

DOG NO.	DATE	WEIGHT IN KILOGRAMS	AVERAGE R.M.E./HOUR, CALORIES	BASAL R.Q.	R.Q. AFTER GLYCINE	TOTAL CALORIES AFTER GLYCINE	TOTAL S.D.A., CALORIES	BLOOD UREA N BEFORE AMYTAL	BLOOD UREA N BEFORE GLYCINE	BLOOD UREA N AT END	EXCESS URINE UREA N	CALORIES/MILLI- MOI. GLYCINE DEAMINIZED
3	6/21/35	5.9	10.09	0.781	0.829	78.59 <sup>1</sup>	18.05	...	17.2	18.8	1088.3	0.23
32	10/28/35	6.1	14.69	0.722	0.788	83.10	9.65	10.7	11.1	16.3	939.9	0.15
31	10/25/35	7.5	15.68	0.779	0.836	89.75	10.35	15.4	8.1	9.1	259.7	0.45
4	6/25/35	8.4	19.57	0.783	0.800	136.33 <sup>1</sup>	18.91	...	30.0	19.0		
29	10/22/35	9.9	12.47	0.813	0.825	70.56	9.69	9.4	9.5	15.2	600.4	0.23
28	10/18/35	11.3	16.88	0.816	0.801	94.26	9.82	11.8	10.7	8.3	573.1	0.24
Av.				0.782	0.813		12.75		14.4	14.5		0.22

<sup>1</sup>Six hours.

## RESULTS

In table 1 we have summarized the results of the injection of 10 gm. of glycine into each of six dogs. In all except dog 28, where the R.Q. was already high, the R.Q. rose. Heat

production also was increased. We are unable to explain why the S.D.A. should be so high in dogs 3 and 4, but when calculated on the basis of millimols of glycine metabolized dog 3 shows a value of 0.23 Cal. per millimol which is close to the average of 0.22. Since dog 4 started with such a high blood urea nitrogen (30 mg. %) and at the end of the experiment showed a figure so much lower (19.0 mg. %), we were unable to calculate the increase heat production in Calories/millimol, with any degree of accuracy. The value of 0.45

TABLE 2  
*A typical experiment. Dog 42, weight 6.4 kg., 11/19/35*

TIME	B.M.R. CALORIES/HOUR	R.Q.	BLOOD UREA N MG./PER CENT	URINE UREA N/HOUR MG.
9.35-9.55	13.72	0.710	7.6	
10.02-10.25	13.94	0.712		
10.32-10.52	13.98	0.731	6.0	68.6
Bilateral nephrectomy 11.04-11.13				
11.17-11.37	12.22	0.704		
11.43-12.03	12.63	0.734		
12.07-12.37	12.59	0.759	7.9	
7.88 gm. glycine intravenously 12.45-1.05				
1.10-2.05	16.10	0.798	16.1	
2.12-3.00	15.26	0.819	21.9	
3.07-4.02	14.94	0.778	27.5	
4.07-5.00	14.19	0.743	30.0	
5.05-6.00	13.04	0.751	36.9	

Average B.M.R. after nephrectomy 12.48 Cal.

S.D.A. of glycine 11.13 Cal.

Cal./millimol seen in dog 31 is probably somewhat higher than the real increase should be. During the control period the blood urea nitrogen fell from 15.4 mg. % to 8.1 mg. %, which resulted in the elimination of considerable urea nitrogen over and above that actually formed during the period. As the control values were deducted from the total excreted during the experimental period, our deduction in this case was undoubtedly too great.

Table 2 shows a typical nephrectomy experiment. The main points to be emphasized here are the constancy of the

basal metabolism under sodium amytal, both before and after nephrectomy; the curve of the increased heat production after the injection of glycine; the increase in the R.Q. after glycine; and the accumulation of urea nitrogen in the blood stream at a practically constant and uniform rate.

TABLE 3  
*S.D.A. of nephrectomized dogs (7.88 gm. glycine)*

DOG NO.	DATE	WEIGHT OF DOG IN KILOGRAMS	AVERAGE R.M.R. BEFORE INJE- CTION CALORIES / HOUR	AVERAGE R.Q. BEFORE INJECTION	AVERAGE R.Q. AFTER INJECTION	TOTAL CALORIES AFTER INJE- CTION	S.D.A., CALORIES	BLOOD UREA N BEFORE INJECTION	BLOOD UREA N AT END	BASAL URINE UREA N EXCRETION / HOUR
45	11/22/35	5.2	8.01	0.745	0.817	53.52	13.47	8.8	44.4	17.7
50	12/17/35	6.1	10.14	0.734	0.831	63.50	12.80	7.9	33.9	56.0
38	11/ 8/35	6.4	11.67	0.793	0.876	69.03	10.68	7.4	26.7	75.7
39	11/11/35	6.4	11.09	0.754	0.802	70.98	15.53	11.7	50.0	81.3
42	11/19/35	6.4	12.48	0.732	0.778	73.53	11.13	7.9	36.9	68.6
46	12/10/35	6.4	10.63	0.746	0.800	68.66	15.51	14.4	52.7	91.8
43	11/20/35	6.8	11.69	0.830	0.890	72.91	14.46	29.2	44.1	87.5
47	12/11/35	8.2	14.90	0.761	0.835	90.68	16.18	10.8	38.1	126.1
48	12/13/35	8.2	12.26	0.807	0.818	83.35	20.05	11.3	27.5	32.8
36	11/ 6/35	8.4	12.75	0.747	0.856	79.12	15.37	7.2	29.9	60.2
51	12/20/35	8.6	15.47	0.755	0.783	86.40	11.27	13.6	38.3	189.3
44	11/21/35	10.2	15.75	0.782	0.745	77.56 <sup>1</sup>	15.33 <sup>1</sup>	13.4	38.8	29.8
35	11/ 5/35	10.7	15.91	0.853	0.858	96.36	16.81	17.5	51.5	411.6
40	11/12/35	13.2	17.46	0.741	0.763	105.09	17.79	14.6	49.3	184.6
Av.				0.770	0.818		14.74	12.5	40.1	108.1
Controls—115 cc. 0.9% NaCl given instead of glycine										
53	12/24/35	5.5	9.92	0.804	0.815	29.63 <sup>2</sup>	0.13	19.7	23.1	68.3
55	12/30/35	6.4	9.18	0.759	0.809	38.56 <sup>2</sup>	1.84	15.3	18.4	56.4
54	12/27/35	7.5	11.58	0.707	0.730	48.50 <sup>1</sup>	2.18	14.5	21.8	66.4
Av.				0.757	0.785		1.30	16.5	21.1	63.7

<sup>1</sup> Four hours.

<sup>2</sup> Three hours.

The summarized results of the injection of glycine into nephrectomized dogs are shown in table 3. There is again an increase in the R.Q. occurring in all dogs except dog 44. The specific dynamic action of glycine is, if anything, slightly

higher than in the control series. Blood urea nitrogen increased markedly averaging more than a threefold increase over the 5-hour period.

In the three dogs injected with only sodium chloride solution, heat production was but slightly increased. If one deducts this slight average increase from the value obtained in the nephrectomized dogs, the average net specific dynamic action of the glycine amounts to 13.44 Cal. in contrast to 12.75 Cal. for the control series.

#### DISCUSSION

Our results show that it is practicable to study the specific dynamic action of glycine in the dog anesthetized with sodium amytal. When expressed in Calories per millimol of glycine metabolized they are in accord with the values of Wilhelmj and Mann ('30); but in our experiments the amount of glycine metabolized was somewhat less. Since the specific dynamic action of glycine can be studied after sodium amytal it is possible to determine the effect of glycine immediately after nephrectomy. This is particularly desirable because it eliminates to a large degree any complication of results that might result with the large accumulation of urea or other metabolic end products. The results on the nephrectomized dogs receiving only 0.9% sodium chloride seem to us to be sufficient evidence that our results are not greatly complicated by these factors.

It seems worth while to point out that our results confirm those of Chambers and Lusk ('30) that the specific dynamic action of glycine is, at least to a large extent, independent of the size of the dogs used.

Space does not permit an adequate discussion of the various theories of specific dynamic action of amino acids. Borsook ('36) had adequately reviewed them and attempted to combine them into a theory which is in accordance with the known facts. Our results on the unoperated dogs agree with his ('35) in so far as the correlation between excess Calories and excess urinary nitrogen are concerned. We

believe he ('31 a, '31 b, '31 c) has much over estimated the role of the kidney in the specific dynamic action of amino acids. We do not intend to imply that the kidney does no work in the concentration of the urine or the excretion of the excess urea, but our results do show that this work is so small when compared with the total heat production as to be, for all practical purposes, unmeasurable even when accurate methods are used.

### CONCLUSIONS

1. The specific dynamic action of glycine, when expressed on the basis of Calorie per millimol deaminized, is the same when determined under sodium amytal anesthesia as in the unanesthetized dog.

2. The specific dynamic action of glycine is independent of the size of the dog, at least within the limits of those used in this investigation.

3. The kidney is not responsible for any measurable amount of the specific dynamic action of glycine.

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# PHOSPHORUS DEFICIENCY METABOLISM AND FOOD UTILIZATION IN BEEF HEIFERS <sup>1</sup>

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## 1. INTRODUCTION

The respiration trials reported in this paper were carried out in order to determine the influence of phosphorus deficiency on the efficiency of food utilization. The term 'efficiency of food utilization' may have several different meanings and is often used rather vaguely. It is necessary therefore to define the term.

*Weight efficiency* relates gain in body weight to weight of the food consumed. In this paper we are mainly dealing with energy efficiency, a relation between the chemical energy of the animals' products and the chemical energy in the food. By energy in the animals' product, or net energy N, we mean the heat of combustion of the gained body substance (or the heat of combustion of the produced milk plus the heat of combustion of the gained body substance etc.). The net energy is expressed in kilogram calories or in therms (1 therm = 1000 kg. calories).

The food energy may be expressed as heat of combustion of the food, as digestible energy or as metabolizable energy. The following formulation is applicable to any of these three bases for measuring food energy. The calculations of our

<sup>1</sup> This report is part of an investigation on the relation of nutrition to reproduction which became cooperative with the United States Bureau of Animal Industry, July 1, 1929.



results (tables 8, 9, 10) are based on metabolizable energy but table 5 furnishes the data necessary for expressing the results also in terms of digestible food energy or heat of combustion of the food. The unit for the food energy is the same as that for the net energy.

Two main types of efficiency should be distinguished, the total efficiency and the partial efficiency.

The total efficiency is the net energy per unit of the energy (heat of combustion or digestible energy or metabolizable energy) in the total food, thus:

$$\text{Total efficiency} = \frac{\text{Net energy}}{\text{energy in total food}} = \frac{N}{U} \quad (1)$$

At a certain food level (maintenance level) the net energy will be zero,  $N = 0$  (that does not mean that nothing is produced but that the sum of the energy in the product is zero, a milk cow, for example, may continue to yield milk but at the cost of her own body substance). The total efficiency at the maintenance level is zero. If the food ration is smaller than the maintenance requirement, the net energy, and consequently also the total efficiency are negative. The total efficiency characterizes the economy of animal production. It does not characterize the nutritive value of the food. In order to measure the food value it is necessary to eliminate the influence of the maintenance requirement of the animal. This can be done by feeding the animal at two different levels and determining the difference in net energy resulting from the difference in the food energy; thus one measures the effect of a part of the food energy and may formulate:

$$\text{Partial efficiency} = \frac{\text{Difference in net energy}}{\text{Difference in food energy}} = \frac{N_1 - N_2}{U_1 - U_2} \quad (2)$$

Below maintenance both  $N_1$  and  $N_2$  will be negative and the partial efficiency will, as a rule, be positive even though the total efficiency is negative.

If the partial efficiency is independent of the plane of nutrition ( $\frac{N_1 - N_2}{U_1 - U_2} = k$ ) then the net energy  $N$  will be proportional to the difference of the total food energy  $U$  and the maintenance requirement  $M$ .

$$N = k (U - M) \quad (3)$$

In this case the following equation shows the relation between partial efficiency  $k$  and total efficiency  $\frac{N}{U}$ .

$$\frac{N}{U} = k \left(1 - \frac{M}{U}\right) \quad (4)$$

In order to formulate the relation of the total efficiency to the fasting katabolism  $B$  instead of the maintenance requirement  $M$  it may be considered that for maintenance  $U_1 = M$  and  $N_1 = 0$  and for fasting  $U_2 = 0$  and  $N_2 = -B$

$$k = \frac{N_1 - N_2}{U_1 - U_2} = \frac{0 - (-B)}{M - 0} = \frac{B}{M} \quad (5)$$

If this result ( $M = \frac{B}{k}$ ) is introduced into equation (4) one obtains for the total efficiency

$$\frac{N}{U} = k - \frac{B}{U} \quad (6)$$

The result of this equation may be stated as follows:

If the partial efficiency is independent of the plane of nutrition then the total efficiency is the difference between the partial efficiency and the quotient  $\frac{\text{Fasting katabolism}}{\text{Total energy intake}}$ .

The reciprocal of this quotient, namely  $\left(\frac{U}{B}\right)$ , is a measure for the level of food intake (relative food level) indicating how many times as much energy the animal takes in as it spends during fasting. If the animal can eat as much as it wants then the quotient  $\frac{U_a}{B}$  characterizes the appetite of the animal.

The presupposition of a constant partial efficiency is strictly applicable only in special cases. The general formulation of the total efficiency (with variable partial efficiency)

is more complicated.<sup>2</sup> The definition of the terms by equation (6) is, however, sufficiently precise for the discussion in this paper.

An influence of phosphorus deficiency on the efficiency of food utilization has been observed by Theiler in his classical investigation on aphosphorosis in South Africa. Theiler ('33) concluded that animals given sufficient phosphorus utilize their food better and gain more per unit of food consumed than do animals on a phosphorus-low diet. Theiler thus observed that phosphorus deficiency lowers the total weight efficiency of the animals.

Eckles and Gullickson ('27) concluded from their experiments that cows on a phosphorus-low diet needed at least 20% more digestible nutrients to maintain their live weight than was indicated by Morrison's feeding standard.

Riddell, Hughes and Fitch ('34) studied the oxygen consumption of cows kept alternately at normal and low phosphorus intake. From the 6-minute records, obtained by

<sup>2</sup>In general the partial efficiency varies with variations in the plane of nutrition. It is often higher for submaintenance than for super-maintenance feeding. If  $k_p$  stands for the partial efficiency above maintenance and  $k_m$  for the partial efficiency below maintenance then the total efficiency may be expressed as follows:

$$\frac{N}{U} = k_p - \frac{k_m B}{k_m U} \quad (6')$$

If the partial efficiency varies even within the submaintenance and super-maintenance levels as indicated by the result of Forbes et al. ('28), Wiegner and Ghoneim ('30), Mitchell et al. ('32) and confirmed by the experiments reported in this paper (p. 150) then the best characteristic for the nutritive value of the food would be the differential efficiency  $\frac{dN}{dU} = \eta$  and in this case the total efficiency may be formulated as follows:

$$\frac{N}{U} = \frac{\int_0^U \eta dU}{U} - \frac{B}{U} \quad (6'')$$

The term  $\left( \frac{\int_0^U \eta dU}{U} \right)$  may be classified as the mean partial efficiency for the entire food level and the result summarized as follows:

In general the total efficiency is the difference between the mean partial efficiency of the entire food and the quotient  $\frac{\text{Fasting katabolism}}{\text{Total energy intake}}$ .

Brody's muzzle method 12 hours after the last food was given, they selected those that were most uniform and showed the lowest oxygen consumption. These authors concluded that the addition of phosphorus to a phosphorus-low diet lowers the general metabolism. The main criticism applying to their result is that the metabolism of cows 12 hours after the last feeding is neither a true fasting katabolism nor representative of the average daily metabolism of the fed animal.

The results reported in the present paper are based on thirty-four complete metabolism trials conducted with three pairs of beef heifers fed all the same diet low in phosphorus but one heifer of each pair receiving a supplement of phosphate. Each metabolism trial lasted 2 weeks. The fasting katabolism was determined in sixteen respiration trials of 48 hours duration during the fourth and fifth day of fasting.

The results of our experiments confirm the observation of previous investigators that phosphorus deficiency lowers the efficiency of food utilization for gain in weight, more specifically the total weight efficiency. The new information supplied by our work is the influence of phosphorus deficiency on the gain or loss of body substance in terms of chemical energy, the energy efficiency, and the differentiation of the total energy efficiency in the two components: partial efficiency and appetite, the appetite being determined as the quotient

$$\frac{\text{Intake of food energy at abundant feeding}}{\text{fasting katabolism}}$$

## 2. METHOD

*Animals used.* Six beef heifers were selected and matched into pairs, their weights, their gains from birth, and also the weights of their ancestors being considered. Animals 18, 27 and 29 were pure bred Herefords; nos. 10 and 51 were pure bred Aberdeen-Angus; and no. 32 was a crossbred Aberdeen-Angus-Shorthorn. All the animals had been raised in the university herd. Only four were kept under observation throughout the entire experimental period. The animals were 14 to 15 months of age when started on the experiment.

*Food and feeding.* The guiding principle in selecting rations was to secure one deficient in phosphorus, yet complete with regard to other nutrient constituents. This is accomplished only with difficulty, particularly in providing adequate protein, when a roughage good in protein and low in phosphorus is not available. The rations used, together with the chemical composition, are shown in table 1.

TABLE 1  
*Composition of food per 100 gm. as fed*

	RATION 1 CONTROL	RATION 2 LOW-PHOS- PHORUS	RATION 3 CONTROL	RATION 4 LOW-PHOS- PHORUS	RATION 5 CONTROL	RATION 6 LOW-PHOS- PHORUS
	gm.	gm.	gm.	gm.	gm.	gm.
a. Food mixture						
Alfalfa molasses meal	30.0	30.0	30.0	30.0		
Dried beet pulp	33.0	30.0	30.0	30.0	62.0	62.5
Cornstarch	25.0	25.6	31.9	32.6	30.0	30.0
Casein	2.5	2.5	1.5	1.5	1.5	1.5
Corn gluten meal	7.5	7.5				
Cottonseed oil	1.5	1.5	3.0	3.0	2.5	2.5
Cod liver oil	0.5	0.5	0.5	0.5	1.0	1.0
Calcium carbonate	1.0	2.4	1.0	2.4	1.0	2.5
Calcium acid phosphate	2.0		2.1		2.0	
b. Chemical analyses						
Moisture	10.20	10.00	13.20	12.20	13.40	13.20
Crude protein	13.38	13.97	8.27	8.84	6.43	6.45
Fat	1.75	1.79	2.53	2.57	0.75	0.80
Ash	6.08	5.12	6.17	5.62	4.88	4.30
Crude fiber	11.72	12.06	11.80	11.05	9.94	10.54
N. F. E.	56.37	57.06	58.03	59.72	64.60	65.31
Calcium	1.57	1.43	1.48	1.52	1.28	1.29
Phosphorus	0.51	0.13	0.45	0.09	0.41	0.068
Carbon	37.68	38.20	38.40	38.00	38.20	39.20
Calories per gram	3.88	3.92	3.78	3.79	3.75	3.68

Cottonseed oil was used in the rations to reduce the dryness and dustiness caused principally by the cornstarch and to make the physical characteristics of the rations acceptable to the animals. The oils were first mixed with the beet pulp or beet pulp and alfalfa meal in a power mixer; then the cornstarch and other ingredients were added and mixed. The finer components adhered to the alfalfa meal and to the beet pulp so that the mixture remained uniform.

All animals were started on ration 1 on January 16, 1932, and continued until February 27, 1932, when no. 32 was changed to ration 2 (low in phosphorus). The remaining five animals continued on ration 1 throughout respiration trials 1 to 6 inclusive. Animals 51 and 27 were changed to low-phosphorus ration 2 on May 14th and May 28th, respectively, and were on this ration during respiration trials 7 to 9, inclusive. During July and August, 1932, control animals 10, 18 and 29 were changed to ration 3, and the low-phosphorus animals 27, 32 and 51 were changed to ration 4, the animals being on these rations during respiration trials 13 to 16, inclusive, and remaining until September 27, 1933. On this date heifers 18 and 29 were changed to control ration 5, and heifers 27 and 32 to phosphorus-low ration 6. The animals remained on these rations until July 26, 1934, when they were slaughtered. Shortly after nos. 18 and 29 were changed to ration 5, a monocalcium phosphate especially prepared for animal feeding was substituted for dicalcium phosphate. Soon thereafter the animals went off feed and did very poorly for about 6 weeks. It was discovered that something in the mineral supplement, presumably ferrous compounds, caused the fats in the ration to become rancid within 24 hours after mixing. As soon as dicalcium phosphate feeding was resumed, the appetite of the animals returned to normal.

Animals 10 and 51 were taken out of the experiment on July 18 and September 28, 1933, respectively.

The animals were fed individually twice each day during most of the experimental period. Feeding thrice daily was tried for a time to see whether or not a significantly higher feed intake could be attained. Except at feeding time during the early part of the experiment the animals all ran together in a small paved corral adjacent to their stalls. During the latter part of the experiment the controls were entirely separated from the low-phosphorus animals to prevent the latter from consuming phosphorus-rich feces of the controls.

The amounts of dry matter consumed per day during each metabolism trial are presented in table 5. During the first

nine trials the two pair mates were fed equal amounts of food at approximately one-half, one and one and one-half maintenance levels. After 1 year of experiment the animals on the P deficient diet would not even eat enough for maintenance (trials 13 to 16) equal amounts of food were still fed to the pair mates at the one-half maintenance level.

After 2 years of the P-low regimen the difference in weight between the animals of one pair became so great that equal amounts of food for both no longer represented the same food level. Food levels are not comparable when they are expressed in kilograms or calories per animal or per kilogram of the animals' weight but are comparable when given in terms of the energy requirement (for example one-half maintenance, maintenance, etc.). The maintenance requirement is approximately proportional to the fasting katabolism of the animal (see equation (5), p. 123) consequently the food level may also be expressed in terms of the fasting katabolism (equation (6)). The fasting katabolism in turn is proportional to the  $\frac{3}{4}$  power of body weight (Kleiber, '32), therefore the food level may also be expressed in terms of the  $\frac{3}{4}$  power of body weight and two animals on the same diet may be regarded as being on the same relative food level if their food intakes per kg.<sup>3</sup> are equal.<sup>3</sup> Based on this consideration we made the rations in trials 22 and 23 so that the pair mates were given approximately the same amount of food per kg.<sup>3</sup>. The higher rations were measured according to the same principle but the phosphorus deficient animals ate only a fraction of these rations.

*Methods of analysis.* Composite samples of the feed used were taken for chemical analysis from a large batch of the mixed feed as the daily rations were being weighed out for the respiration trials. These composites were then run through a Wiley mill, reduced in size by quartering, and finally ground to pass a 40-mesh sieve. In the first six trials the total feces voided each day were dried in a large

<sup>3</sup> The symbol  $\text{kg.}^{\frac{3}{4}}$  is used throughout this paper as unit of the  $\frac{3}{4}$  power of body weight,  $W^{\frac{3}{4}}$ , where W is expressed in kilograms.

electric oven at 90° to 95°C. The dry residues were weighed and added together until the end of the 12-day trial. The total dry matter voided was thus determined. The dry feces were then run through a laboratory hammer mill, and samples obtained for chemical analysis.

When, later, it was found that this method of drying feces caused a loss of some carbon and nitrogen, a different procedure was adopted. The moist feces from each 24 hours were carefully mixed, and an aliquot was taken for total nitrogen and for moisture and volatile carbon compounds. This procedure has been described by Kleiber, Caldwell and Johnson ('36). The remaining fresh feces were then dried as before in the air oven at 70°C. and a composite dry sample was prepared for analysis. Corrections were then applied for losses of C during drying. The value used for the total nitrogen in the feces was that obtained from the analysis of the wet feces samples taken daily.

All feed and feces samples were analyzed for crude protein, fat, ash, crude fiber and moisture, according to Methods of Analysis, Association of Official Agricultural Chemists, 1930 edition. Calcium was determined on the ash by McCrudden's method ('10, '11). Phosphorus was determined colorimetrically by the method of Fiske and Subarrow ('25), modified for ash materials. The accuracy of this method for feed and feces phosphorus was repeatedly checked by the official volumetric method of the Association of Agricultural Chemists ('30).

Blood calcium was determined by the Clark-Collip ('25) method, and inorganic serum phosphate by the Fiske and Subarrow ('25) method.

The fuel value of feeds and feces was determined in an Emerson fuel calorimeter. Carbon was determined after each combustion by allowing the CO<sub>2</sub> to escape from the bomb through an absorbing tower containing BaCl<sub>2</sub>—Ba (OH)<sub>2</sub> solution. Titration of the excess Ba(OH)<sub>2</sub> was made with a mixed indicator, described in detail by Kleiber ('35).



Carbon determination on urine was carried out by a wet-combustion process as described by Mohlman and Edwards ('31). Instead of using a mixture of  $\text{H}_3\text{PO}_4$  and  $\text{H}_2\text{SO}_4$  and a solution of chromic acid, a mixture of  $\text{K}_2\text{Cr}_2\text{O}_7$  and  $\text{NaH}_2\text{PO}_4$  was made up. The proportions used were 7 gm.  $\text{K}_2\text{Cr}_2\text{O}_7$  to 25 gm.  $\text{NaH}_2\text{PO}_4$ . These amounts are the quantities used per determination of approximately 100 mg. carbon. In place of the mixture of  $\text{H}_3\text{PO}_4$  and  $\text{H}_2\text{SO}_4$ , 90 cc. of concentrated  $\text{H}_2\text{SO}_4$  were added. The method was tried on benzoic acid, sulphanilic acid and sodium formate. It produced 98.0, 99.1 and 99.0% recovery of the C, respectively.

The analyses of the bones for  $\text{CaCO}_3$  were carried out on samples of dry, fat-free bones; for the determination of Ca and phosphorus the bone samples were ashed.

*Respiration trials: Procedure.* In order to determine the partial efficiency of energy utilization one must determine the energy balance at two different levels of food intake (see equation (2) of introduction). One of these levels may be at complete fasting. Earlier observations of Forbes and co-workers ('28) as well as Mitchell and collaborators ('32) have indicated that the partial efficiency of energy utilization in cattle may depend on the food level. It seemed desirable, therefore, to run trials with food levels below as well as above maintenance. The appetite of our animals on phosphorus-low rations, however, was so poor that the plan of studying the energy utilization at high food levels had to be abandoned. We have not even been successful in every case in keeping the low-phosphorus animals at a maintenance level.

The history of the  $2\frac{1}{2}$ -year series of respiration trials with the beef heifers is shown in table 5. Each respiration trial for the animals except those on basal metabolism studies lasted 2 weeks, of which time two 5-day periods constituted uninterrupted respiration experiments. The average coefficient of variation of the daily  $\text{O}_2$  consumption within one trial amounted to  $\pm(4.4 \pm 0.8)\%$  for the phosphorus deficient and  $\pm(4.2 \pm 0.6)\%$  for the control heifers. The corresponding figures for the  $\text{CO}_2$  production are  $\pm(4.5 \pm 0.91)\%$  for

the phosphorus deficient and  $\pm(1.8 \pm 0.25)\%$  for the control heifers. The average standard deviation of the daily R.Q. within one trial was  $\pm(0.029 \pm 0.006)$  for the phosphorus deficient and  $\pm(0.028 \pm 0.007)$  for the control heifers. The measurements of the fasting katabolism were made in a 48-hour experiment during the fourth and fifth day after the last food.

The technic of the respiration trials followed at this station has been described in detail by Kleiber ('35).

### 3. SYMPTOMS OF PHOSPHORUS DEFICIENCY

*Gain in weight.* The weight curves of the animals essentially confirm the earlier observations concerning the effect of phosphorus deficiency on growth. During the first 6 months after changing to phosphorus-low rations 2 and 4, heifers 27, 32 and 51 continued to gain at nearly the same rate as their controls on rations 1 and 3. At the end of this time the low-phosphorus heifers ceased to gain; their weights remained about constant for a period of approximately a year. During this time the controls continued to gain. After the change to rations 5 and 6 the control animals maintained their weight while the weight of the low-phosphorus animals declined. In the following discussion the period of the first 6 months (apparently no influence of phosphorus-deficient food on growth) is termed the first period of the experiment. The following year (cessation of growth in phosphorus-deficient animals) is classified as the second, and the last half-year (decline in weight of the phosphorus-deficient animals) as the third period of the experiment. The difference between the initial and final weight of control animal 18 was 203 kg. that of control animal 29 was 190 kg., while the corresponding differences between initial and final weights of the phosphorus-deficient animals 32 and 27, was only 36 and 6 kg., respectively. Some evidence of limitation of skeletal growth is afforded by the fact that control animals 18 and 29 increased in height at withers 9.5 and 11 cm., respectively, while their low-phosphorus mates increased but 6.5 and 8.5 cm. in height, respectively.

*Concentration of phosphorus in the blood serum.* The data on serum phosphorus are presented in table 2. Most of the data are based upon composite samples collected on each of 2 or 3 successive days. A few determinations based upon single samples are included. The control animals maintained high inorganic phosphorus content of the blood serum

TABLE 2  
*Content of inorganic phosphorus in blood serum<sup>1</sup>*

DATE	MILLIGRAMS PHOSPHORUS PER 100 CC. BLOOD SERUM					
	Control heifers			Low-phosphorus heifers		
	No. 10	No. 29	No. 18	No. 32	No. 27	No. 51
2-25-32	8.9 (1)	(1)	10.1 (1)	9.02 (1)	(1)	9.6 (1)
3-12-32	8.4	8.3		6.1 (2)	11.1	
3-19-32	7.9			6.8		
3-26-32	6.7		9.4	6.0		8.1
4- 2-32	9.1			7.0		
4- 7-32	9.3	9.2		6.7	10.4	
4-28-32			10.0			8.8
5-12-32	7.9	8.6		6.1	8.8 (2)	
6- 3-32	8.2			4.7		
6- 9-32	8.2		10.3	7.8		7.2 (2)
6-18-32	9.2			8.2		
7- 6-32	10.9 (3)	9.7 (3)	11.2 (3)	7.8 (4)	6.2 (4)	7.5 (4)
7-23-32	7.6	9.8	10.5	6.5	7.4	7.4
8- 6-32	7.9			7.3		
8-20-32	8.1	8.4	11.2	5.7	5.6	7.2
9- 2-32				4.4	4.2	5.1
9-10-32	7.8	8.7		5.1	5.5	5.5
9-17-32	8.4	9.3	8.2	6.5	4.7	6.6
10- 6-32	6.7	8.6	9.8	5.1	4.8	5.7
11-17-32	8.8	8.9	10.3	4.6	4.4	6.9
1-12-33	7.8	8.0	9.6	5.5	3.9	5.6
3-25-33	7.5	8.6	10.0	4.3	4.5	5.3
4-15-33	8.3	8.9	9.7	4.8	4.2	5.1
6-16-33		8.5	9.5	5.1	4.9	4.9
9-27-33		(5)	(5)	3.6 (6)	3.1 (6)	
12-19-33				4.6	4.4	
2-12-34		6.9	8.6	2.3	2.8	
3-24-34		7.9	8.7	3.5	3.7	
5-11-34		6.4	8.1	5.3	4.8	
7-25-34		9.4	11.3	3.5	3.9	
Average	8.27	8.56	9.79			

<sup>1</sup> The figures in parenthesis in the table indicate the ration fed and the date on which the change in ration was made.

throughout the experiment, the range being from 6.4 to 11.3 mg. phosphorus per 100 cc. of serum. Considering the age of the animals, the average values of 8.27, 8.57 and 9.8 mg. phosphorus per 100 cc. serum for nos. 10, 29 and 18, respectively, are somewhat higher than the 'normal' figures usually found (Palmer, Cunningham and Eckles, '30), (Guilbert, unpublished data) and presumably reflect the high phosphorus content of the control rations.

After the change from ration 1 to phosphorus-low ration 2, the blood phosphorus of animal 32 dropped from 9 to an average of 6.77 mg. phosphorus per 100 cc. of serum; after the change to ration 4 the average value was 5.42 mg. phosphorus; and during the period of ration 6 the average value dropped to 3.83 mg. phosphorus per 100 cc. The average values for animal 27 during the periods on rations 4 and 6 were 5.05 mg. phosphorus and 3.95 mg. phosphorus per 100 cc. of serum, respectively. The values for animal 51 during the period on ration 4 averaged considerably higher than those for the other animals on the same feed. The consistently high values found for control animal 18 are also noteworthy.

Although the serum phosphorus values decreased in relation to the phosphorus content of the rations fed, only a few of the determinations during the latter part of the experiment would be indicative of phosphorus deficiency on the basis of the criteria of Palmer, Cunningham and Eckles ('30). The vitamin D from the cod liver oil fed to our animals throughout the experiment may have prevented a more conspicuous fall in blood phosphorus. The range in blood-phosphorus values of dairy heifers on phosphorus-low rations reported by Van Landingham, Henderson and Bowling ('35) are somewhat similar to ours.

*Pica.* All the low-phosphorus animals began showing depraved appetites about December, 1932, coincident with cessation of increase in weight. It was manifested largely by coprophagy, chewing mangers and the chains by which the animals were tied at feeding time. After coprophagy started,

the stalls and corral were swept three times daily and washed with water frequently to prevent the accumulation of dried feces from the control animals, from which the low-phosphorus animals could obtain a significant addition to their phosphorus intake. Notwithstanding these precautions the low-phosphorus animals may at times have obtained added phosphorus in this manner, and this accident may have caused some of the variations found in the serum phosphorus values.

By April, 1933, heifers 32 and 27 showed marked osteophagia when tested with sun-bleached bones. Animal 51 at this time manifested only mild interest, while none of the controls would touch the bones. At this time the serum phosphorus was from 4.20 to over 5.0 mg. phosphorus per 100 cc. of serum. Heifer 51 exhibited definite osteophagia in August, 1933. Animals 32 and 27 were then still in good and animal 51 in excellent condition. As the experiment progressed, pica became more pronounced. All the low-phosphorus animals eagerly licked up blood spilled on the cement floor when blood samples were being taken.

No depraved appetite was noted in the control animals until after the change to ration 5. Wood chewing and occasional licking of urine from the concrete pavement were noted. In other experiments at this station wood chewing has been observed in connection with 'no-roughage' rations regardless of the completeness of the ration in other respects. The licking of urine associated with low-protein intake, has been observed also in other experiments by one of us (H. R. Guilbert).

*Lack of appetite.* Lack of appetite described by earlier workers (see for example, Huffman, Duncan, Robinson and Lamb, '33) was one of the most conspicuous symptoms in our phosphorus deficient animals.

Table 3 shows the maximum of the weekly food intake per kg.<sup>3</sup> during the preliminary period when all animals received a phosphate supplement and during each of the subsequent periods when animals 27 and 32 were on phosphorus-low rations. The reason for expressing the appetite as food intake per unit of the  $\frac{2}{3}$  power of weight has been discussed before (p. 128).

The phosphorus-deficient animals decreased the maximal food intake per kg.<sup>2</sup> consistently as the phosphorus in the food decreased. From the first to the second period the low-phosphorus animals lost about one-fifth of their appetite, while the appetite of the control animals increased slightly.

During the last period of the experiment the maximal food intake per kg.<sup>2</sup> of the two low-phosphorus animals approximated a level one-half of that of the first period. Only a small part of this drastic loss in appetite may be accounted for by a change in the composition of the food aside from its phosphorus content, as indicated by a 10% loss in appetite

TABLE 3  
*Appetite of phosphorus-deficient and control animals*

ANIMALS	MAXIMAL INTAKE OF AIR-DRY FOOD PER WEEK PER UNIT OF BODY SIZE			
	Preliminary period	Time of phosphorus deficiency		
		0-6 months	6-18 months	18-24 months
	kg./w <sup>2.1</sup>	kg./w <sup>2</sup>	kg./w <sup>2</sup>	kg./w <sup>2</sup>
Phosphorus-deficient animals				
No. 27	0.437	0.470	0.404	0.260
No. 32	0.465	0.490	0.364	0.234
Control animals				
No. 29	0.481	0.467	0.526	0.367
No. 18	0.407	0.480	0.485	0.473

<sup>1</sup> Heading reads: "kg. of food divided by W to the  $\frac{1}{2}$  power."

of the control animals. Thus our results confirm the observation of earlier workers (Theiler, Riddell) that phosphorus deficiency depresses the appetite of the animals.

*Oestrus cycles.* During the period January 15, 1932, to January 7, 1933 control animals 18 and 29 exhibited fifteen and sixteen periods of oestrus, respectively. During the next year and a half these animals were for the most part kept in separate quarters from the low-phosphorus animals and were not under so close observation as before. Moreover, they were not particularly demonstrative during oestrus, so that doubtless some periods were not recorded. Seven and nine periods, respectively, were recorded during this period. The occurrence of oestrus appeared somewhat irregular after change

to ration 5, in which the protein level was rather low. At autopsy, however, the ovaries of both animals were found to be normal, and they contained recent corpora. The third control animal, 10, had thirteen periods of oestrus during the first 9 months on the experiment. At the end of this time she bred, conceived, and in due time gave birth to a normal calf. This was considered to be evidence that the control rations 1 and 3 were reasonably adequate.

Animal 27 had, between January, 1931, and May, 1932, six periods of oestrus. At the end of this period she was changed to the phosphorus-low ration. Only three periods of oestrus were observed during the next 7 months, and only two additional periods during the next year and a half of the experiment. At autopsy she was found to have one small, quiescent ovary and one cystic ovary. Low-phosphorus animal 32 came into oestrus ten times during the first 9 months. At the end of this time (September 2, 1932) she was bred but did not become pregnant. A bloody discharge from the vagina was noted on January 21, 1933, and oestrus was recorded on October 18, 1933 and January 3, 1934. These three occasions were the only manifestations of oestrus during the entire period of the experiment, after breeding on September 2, 1932. On autopsy the ovaries of this animal were found to be small and quiescent. The cessation of oestrus in both these low-phosphorus animals coincided closely with cessation of gains. Low-phosphorus animal 51 continued to have more or less regular cycles throughout the entire period she was on the experiment. This animal always had a more thrifty appearance than 27 and 32, a better appetite, a higher content of inorganic phosphate in the blood serum. She was removed from the experiment in September, 1933, and was never placed on phosphorus low ration 6.

Cessation or irregularity of oestrus has been associated with phosphorus deficiency by a number of workers. The literature was reviewed by Eckles, Palmer and associates ('35). The experiments reported in their paper cast some doubt as to whether the interference with oestrus is caused

by phosphorus deficiency per se, as their animals on phosphorus-low rations had normal oestrous cycles. From the standpoint of blood and bone analysis and other symptoms, their animals were deficient in phosphorus; but apparently the intake of phosphorus was varied so that total food consumption was not significantly reduced.

*Bone analysis.* According to Neal and Palmer ('31) the ribs may be considered as the most reliable single samples that represent the condition of the skeleton with regard to its chemical composition. The analysis of the dry, fat-free rib bones of our heifers is presented in table 4.

TABLE 4  
*Composition of rib bone of phosphorus-deficient and control heifers*

CONSTITUENTS	CONTENT PER 100 GM. OF FAT FREE DRY BONES			
	Phosphorus-deficient heifers		Control heifers	
	No. 27	No. 32	No. 18	No. 29
Ash	55.4	58.1	63.0	65.4
Ca	21.6	22.5	24.1	25.0
P	9.49	10.05	11.32	11.75
Mg	0.29	0.32	0.54	0.48
CaCO <sub>3</sub>	8.25	8.25	6.15	7.00
Ca <sub>3</sub> (PO <sub>4</sub> ) <sub>2</sub>	46.20	48.90	54.30	56.70
Ratio: Ca <sub>3</sub> (PO <sub>4</sub> ) <sub>2</sub> CaCO <sub>3</sub>	5.6	5.9	8.8	8.1

The bones of the phosphorus-deficient heifers 27 and 32 had a lower ash content and a higher content of CaCO<sub>3</sub> than the bones of their mates, 18 and 29, on the phosphate-supplemented diet. The ratio Ca<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub>:CaCO<sub>3</sub> is considerably lower in the bones of the control heifers. This result confirms the earlier findings of Neal, Palmer, Eckles and Gullickson ('31). In our trials the effect seemed somewhat more pronounced than in the trials of these authors.

#### 4. RESULTS OF METABOLISM TRIALS

The results of the respiration trials carried out with the six beef heifers are summarized in chronological order in table 5.



TABLE 5  
Phosphorus-deficiency and metabolism chronological summary

TRIAL NO.	DATE	ANIMAL NO.	WEIGHT OR ANIMAL	W <sub>2</sub> KE <sub>1</sub>	FEED NUTR P PER DAY	FEED NUTR P PER 100 GM. FEED	P BALANCE: GRAIN P PER DAY	FEED	DIGESTED	NOTABLE	Net	Heat
1	Feb. 29 to 1932	18	317.1	75.0	1207	0.608	+2.08	5.27	4.14	3.06	-2.57	5.65
2	Mar. 14 to 29	29	223.4	76.0	1207	0.608	+1.78	5.27	3.97	3.04	-3.69	6.73
3	Mar. 26	27	352.4	83.5	1207	0.608	-0.77	5.27	4.08	2.97	-3.83	6.80
4	Apr. 9	61	313.4	74.0	2395	0.576	+6.19	10.46	8.66	6.06	-0.18	7.06
5	Apr. 11 to 23	29	327.0	77.0	2395	0.576	+6.66	10.45	8.69	7.11	-0.80	7.41
6	May 2 to 14	18	341.1	79.0	2395	0.579	+8.52	16.93	13.61	11.65	+3.08	8.57
7	May 16 to 28	61	328.4	77.5	4100	0.592	+5.92	17.73	14.63	12.29	+3.29	9.00
8	June 5 to 18	32	354.3	82.0	4425	0.592	+8.21	17.73	14.21	12.34	+3.01	9.33
9	June 18 to 29	10	338.8	79.0	4474	0.592	+3.9	19.33	15.25	13.31	+1.05	12.26
10	July 5 to 16	32	342.5	80.0	3720	0.500	+8.3	19.29	15.63	13.33	+1.72	11.61
11	July 25 to Aug. 6	10	322.9	77.0	1241	0.497	+3.3	10.90	8.83	7.29	-0.46	7.75
12	Dec. 18 to 22	32	341.9	80.8	1239	0.124	+0.42	5.39	4.77	3.81	-2.37	6.18
13	Dec. 21	29	430.7	104.0								8.26
14	Dec. 22 to 24	10	415.0	92.0								8.78
15	Dec. 24	32	495.0	91.0								6.73
16	Mar. 1 to 15	10	464.0	100.0	1600	0.571	+0.8	6.83	5.50	4.28	-4.74	9.02
17	May 13 to 27	32	416.4	92.0	1634	0.112	-0.1	6.95	5.83	4.58	-2.45	7.03
18	May 28 to 31	29	494.0	104.8	1634	0.112	+1.2	6.83	5.42	4.38	-3.35	7.73
19	June 1 to 2	32	442.7	96.5								8.87
20	June 2 to 10	10	442.7	96.5								8.87
21	June 11 to 27	32	442.7	96.5								8.87
22	June 28 to July 1	32	442.7	96.5								8.87
23	June 2 to 10	10	442.7	96.5								8.87
24	June 11 to 27	32	442.7	96.5								8.87
25	June 28 to July 1	32	442.7	96.5								8.87
26	June 2 to 10	10	442.7	96.5								8.87
27	June 11 to 27	32	442.7	96.5								8.87
28	June 28 to July 1	32	442.7	96.5								8.87
29	June 2 to 10	10	442.7	96.5								8.87
30	June 11 to 27	32	442.7	96.5								8.87
31	June 28 to July 1	32	442.7	96.5								8.87
32	June 2 to 10	10	442.7	96.5								8.87
33	June 11 to 27	32	442.7	96.5								8.87
34	June 28 to July 1	32	442.7	96.5								8.87
35	June 2 to 10	10	442.7	96.5								8.87
36	June 11 to 27	32	442.7	96.5								8.87
37	June 28 to July 1	32	442.7	96.5								8.87
38	June 2 to 10	10	442.7	96.5								8.87
39	June 11 to 27	32	442.7	96.5								8.87
40	June 28 to July 1	32	442.7	96.5								8.87
41	June 2 to 10	10	442.7	96.5								8.87
42	June 11 to 27	32	442.7	96.5								8.87
43	June 28 to July 1	32	442.7	96.5								8.87
44	June 2 to 10	10	442.7	96.5								8.87
45	June 11 to 27	32	442.7	96.5								8.87
46	June 28 to July 1	32	442.7	96.5								8.87
47	June 2 to 10	10	442.7	96.5								8.87
48	June 11 to 27	32	442.7	96.5								8.87
49	June 28 to July 1	32	442.7	96.5								8.87
50	June 2 to 10	10	442.7	96.5								8.87
51	June 11 to 27	32	442.7	96.5								8.87
52	June 28 to July 1	32	442.7	96.5								8.87
53	June 2 to 10	10	442.7	96.5								8.87
54	June 11 to 27	32	442.7	96.5								8.87
55	June 28 to July 1	32	442.7	96.5								8.87
56	June 2 to 10	10	442.7	96.5								8.87
57	June 11 to 27	32	442.7	96.5								8.87
58	June 28 to July 1	32	442.7	96.5								8.87
59	June 2 to 10	10	442.7	96.5								8.87
60	June 11 to 27	32	442.7	96.5								8.87
61	June 28 to July 1	32	442.7	96.5								8.87
62	June 2 to 10	10	442.7	96.5								8.87
63	June 11 to 27	32	442.7	96.5								8.87
64	June 28 to July 1	32	442.7	96.5								8.87
65	June 2 to 10	10	442.7	96.5								8.87
66	June 11 to 27	32	442.7	96.5								8.87
67	June 28 to July 1	32	442.7	96.5								8.87
68	June 2 to 10	10	442.7	96.5								8.87
69	June 11 to 27	32	442.7	96.5								8.87
70	June 28 to July 1	32	442.7	96.5								8.87
71	June 2 to 10	10	442.7	96.5								8.87
72	June 11 to 27	32	442.7	96.5								8.87
73	June 28 to July 1	32	442.7	96.5								8.87
74	June 2 to 10	10	442.7	96.5								8.87
75	June 11 to 27	32	442.7	96.5								8.87
76	June 28 to July 1	32	442.7	96.5								8.87
77	June 2 to 10	10	442.7	96.5								8.87
78	June 11 to 27	32	442.7	96.5								8.87
79	June 28 to July 1	32	442.7	96.5								8.87
80	June 2 to 10	10	442.7	96.5								8.87
81	June 11 to 27	32	442.7	96.5								8.87
82	June 28 to July 1	32	442.7	96.5								8.87
83	June 2 to 10	10	442.7	96.5								8.87
84	June 11 to 27	32	442.7	96.5								8.87
85	June 28 to July 1	32	442.7	96.5								8.87
86	June 2 to 10	10	442.7	96.5								8.87
87	June 11 to 27	32	442.7	96.5								8.87
88	June 28 to July 1	32	442.7	96.5								8.87
89	June 2 to 10	10	442.7	96.5								8.87
90	June 11 to 27	32	442.7	96.5								8.87
91	June 28 to July 1	32	442.7	96.5								8.87
92	June 2 to 10	10	442.7	96.5								8.87
93	June 11 to 27	32	442.7	96.5								8.87
94	June 28 to July 1	32	442.7	96.5								8.87
95	June 2 to 10	10	442.7	96.5								8.87
96	June 11 to 27	32	442.7	96.5								8.87
97	June 28 to July 1	32	442.7	96.5								8.87
98	June 2 to 10	10	442.7	96.5								8.87
99	June 11 to 27	32	442.7	96.5								8.87
100	June 28 to July 1	32	442.7	96.5								8.87

*Phosphorus deficiency and digestibility.* The digestibility of the energy of the experimental food was high and uniform throughout the experiment. The standard deviation of the single experiment is  $\pm 3.1\%$  for the deficient animals and  $\pm 2.5\%$  for the controls. The average digestibility of the food energy in eight respiration trials with the phosphorus-deficient animals in the second and third periods of the experiment is  $83 \pm 1.1\%$ . The average digestibility of the food energy in the corresponding experiments in the control animals is  $81 \pm 0.9\%$ . No influence of phosphorus deficiency on the digestibility of protein could be observed. The apparent protein digestibility in the phosphorus-deficient animals was  $46.1 \pm 5.6\%$ ; that of the control animals  $46.4 \pm 2.2\%$ .

*Phosphorus deficiency and urine excretion.* The phosphorus-deficient animals excreted on the average slightly less nitrogen in the urine per day than did the controls. The difference, which is insignificant, may be partly related to the somewhat higher food intake of the normal animals. In the fasting katabolism trials the urine was collected during the fourth and fifth day of fasting, and one-half the amount of the nitrogen excretion thus found was regarded as the daily nitrogen excretion during fasting. The daily nitrogen loss during fasting was higher than the nitrogen loss of the animals during food intake. The daily average nitrogen loss for the phosphorus-deficient animals during fasting was 34.5 gm. nitrogen. The normal animals lost on the average 38.1 gm. nitrogen per day of fasting. There was thus no increase in protein katabolism during fasting of the phosphorus-deficient animals as compared with their mates.

In the urine of the animals receiving food the  $\frac{C}{N}$  ratio ( $\frac{\text{grams C}}{\text{grams N}}$ ) showed a tendency to be increased with increasing plane of nutrition, ranging from 1.5 to 2.4 in the urine of the phosphorus-deficient animals and from 1.3 to 2.6 in the urine of the controls. The average urinary  $\frac{C}{N}$  ratio for the phosphorus-deficient animals is slightly below that of the controls. The difference is, however, negligible.

During fasting the  $\frac{C}{N}$  ratio in the urine dropped to an average of 1.08 for both groups of heifers, a fact indicating that in starvation the composition of the urine of ruminants approaches that of carnivorous animals.

The phosphorus-deficient animals used the digested protein less efficiently for sparing body protein than the control animals. This result follows from the data in table 6, where the partial relative protein katabolism, namely the increase in urinary nitrogen divided by the corresponding increase in digested nitrogen, is calculated for the change from the one-

TABLE 6

*Partial relative protein katabolism ( $\frac{\Delta N \text{ in urine}}{\Delta N \text{ digested}}$ ) for one-half maintenance to maintenance level*

TRIAL NO.	PHOSPHORUS-DEFICIENT ANIMALS						CONTROL ANIMALS					
	Animal	N digested	N in urine	$\Delta N$ digested	$\Delta N$ in urine	$\frac{\Delta N \text{ in urine}}{\Delta N \text{ digested}}$	Animal	N digested	N in urine	$\Delta N$ digested	$\Delta N$ in urine	$\frac{\Delta N \text{ in urine}}{\Delta N \text{ digested}}$
13	27	27.0	24.5	21.0	11.4	0.55	29	35.6	28.8	22.8	4.3	0.19
15	27	6.0	13.1				29	12.8	24.5			
14	32	26.9	22.2	10.8	5.9	0.55	10	32.0	20.7	18.6	-0.7	-0.04
16	32	16.1	16.3				10	13.4	21.4			
20	27	10.7	15.5	6.8	4.6	0.68	29	13.6	10.8	6.9	1.0	0.15
22	27	3.9	10.9				29	6.7	9.8			
21	32	6.3	10.6	2.1	2.0	0.95	18	11.9	12.7	5.8	0.6	0.10
23	32	4.2	8.6				18	6.1	12.1			

half maintenance to the maintenance food level. The average of this quotient for the second and third periods of phosphorus deficiency amounts to  $0.68 \pm 0.10$  gm. nitrogen katabolized per gram nitrogen digested for the phosphorus-deficient animals and to only  $0.10 \pm 0.05$  gm. nitrogen katabolized per gram nitrogen digested for the control animals.

*CH<sub>4</sub> production.* The amounts of CH<sub>4</sub> produced during the second and third periods of the experiment ranged from seventy-two to 161 liters per day for the control animals.

The rate of CH<sub>4</sub> production is strongly influenced by the plane of nutrition. The correlation coefficient for food intake

and  $CH_4$  production amounts to 0.72 for the phosphorus-deficient and 0.92 for the control animals. The effect of food intake on  $CH_4$  production in our trials may be summarized by the following regression equations for the phosphorus-deficient animals:

$$CH_4 = 37 + 32.6 F$$

for the control animals:

$$CH_4 = 29 + 32.9 F$$

where  $CH_4$  = amount of  $CH_4$  produced daily in liters at standard conditions,  $F$  = amount of dry matter of food consumed daily in kilograms.

The metabolizability of the food energy is the factor by which the amount of total food energy (heat of combustion as measured in the calorimetric bomb) is to be multiplied in order to calculate the amount of energy available for meat, fat or heat production. The average metabolizability of the food energy in the phosphorus-low rations of  $67.0 \pm 1.1\%$  is practically equal to the average metabolizability of  $66.6 \pm 1.3\%$  for the energy in the control rations.

*CO<sub>2</sub> production.* Table 7 presents the results on the respiratory exchange of the phosphorus deficient and the control animals during the second and third period of our experiment. The effect of phosphorus deficient food on the respiratory exchange should be compared with the effect of the phosphorus supplemented food. For this comparison it is necessary to summarize the effect of food on the respiratory exchange in all the trials with phosphorus deficient as well as in the trials with the control animals, since the original plan of having a comparison within each pair trial was defeated by the low appetite of the phosphorus-deficient animals. In order to summarize the effect of food intake on the respiratory exchange one may assume a linear relation between the two variables, such as formulated in equation (7)

$$y = y_0 + rx \tag{7}$$

where  $y$  = respiratory exchange,  $y_0$  = respiratory exchange without food,  $x$  = food intake,  $r$  = a constant characterizing the effect of food.

TABLE 7  
*Respiratory exchange of phosphorus-deficient and control animals*

TRIAL NO.	PHOSPHORUS-DEFICIENT ANIMALS					CONTROL ANIMALS				
	Animal No.	Food dry consumed per day <i>gm.</i>	CO <sub>2</sub> produced per day liters (s) <sup>1</sup>	O <sub>2</sub> consumed per day liters (s)	R. Q.	Animal No.	Food dry consumed per day <i>gm.</i>	CO <sub>2</sub> produced per day liters (s)	O <sub>2</sub> consumed per day liters (s)	R. Q.
13	27	2814	1883 ± 29	2015 ± 30	0.958 ± 0.013	29	4331	2222 ± 14	2201 ± 45	1.042 ± 0.018
14	32	2640	1714 ± 40	1705 ± 44	1.013 ± 0.033	10	4331	2282 ± 10	2188 ± 32	1.035 ± 0.005
15	27	1634	1538 ± 24	1812 ± 27	0.871 ± 0.015	29	1600	1441 ± 11	1691 ± 24	0.874 ± 0.007
16	32	1634	1307 ± 25	1468 ± 40	0.915 ± 0.021	10	1600	1633 ± 12	1956 ± 36	0.871 ± 0.013
17	27	0	1129	1834	0.616	29	0	1128	1620	0.711
18	51	0	1237	1783	0.694	18	0	1259	1819	0.709
19	32	0	964	1284	0.704	10	0	1484	2170	0.700
20	27	1656	1513 ± 7	1648 ± 11	0.919 ± 0.007	29	2756	1880 ± 9	1960 ± 14	0.959 ± 0.003
21	32	1179	1158 ± 36	1247 ± 35	0.928 ± 0.013	18	2800	1819 ± 13	1891 ± 29	0.974 ± 0.006
22	27	1209	1310 ± 9	1516 ± 8	0.863 ± 0.003	29	1410	1452 ± 8	1697 ± 14	0.856 ± 0.004
23	32	1203	1100 ± 12	1222 ± 11	0.898 ± 0.003	18	1401	1442 ± 11	1630 ± 27	0.887 ± 0.016
24	27	0	1088	1536	0.708	29	0	1232	1730	0.712
25	32	0	888	1210	0.734	18	0	1075	1478	0.727

<sup>1</sup> Liters (s) = liters at 0°C, 760 mm. Hg pressure and dry.

If  $y_0$  and  $r$  are constant they may be calculated from any number of experiments with varying  $x$  and  $y$ . The respiratory exchange without food,  $y_0$ , can, however, not be regarded as a constant since it depends on the body size of the animals, which varied during the experiment. In order to eliminate the influence of body size we divide the entire equation (7) by the  $\frac{3}{4}$  power of body weight and formulate

$$\frac{y}{W^{\frac{3}{4}}} = \frac{y_0}{W^{\frac{3}{4}}} + r \frac{x}{W^{\frac{3}{4}}} \quad (8)$$

Since the fasting katabolism in calories per  $\text{kg.}^{\frac{3}{4}}$  is constant and since the respiratory exchange at a given R. Q. is proportional to the heat production, the term  $\frac{y_0}{W^{\frac{3}{4}}}$ , the fasting gas exchange per  $\text{kg.}^{\frac{3}{4}}$ , may also be regarded as a constant. The method of least squares (Ezekiel, '30, p. 55) has thus been applied to the relation of gas exchange per  $\text{kg.}^{\frac{3}{4}}$  and food consumption per  $\text{kg.}^{\frac{3}{4}}$ .

The following regression equation resulted from twelve respiration trials with the phosphorus-deficient animals (27 and 32) during the second and third periods of the experiment (table 7):

$$C = 10.7 + 0.248 f \quad (9)$$

where  $C$  = liters  $\text{CO}_2$  produced daily per  $\text{kg.}^{\frac{3}{4}}$ ,  $f$  = grams dry matter of food consumed daily per  $\text{kg.}^{\frac{3}{4}}$ .

The corresponding equation for the two control animals 29 and 18 based on ten respiration trials reads

$$C = 10.6 + 0.233 f \quad (10)$$

The difference in the regression coefficients seems to indicate that the food increases the katabolic processes in the phosphorus-deficient animals to a greater extent than in the control animals. This difference in the regression coefficients in our trials, namely  $0.015 \pm 0.0244$  is, however, not statistically significant, the probability that it occurred by random being between 50 to 60% (Fisher, '30, table 14). To prove this difference according to statistical rule, 105 pair trials would have to be carried out if the standard deviation of a

determination would remain the same as in our experiments and if the mean results did not change.

*O<sub>2</sub> consumption.* The data for O<sub>2</sub> consumption during the second and third period of our experiment are presented in table 7. During the fourth day of fasting the phosphorus deficient animals consumed on the average daily  $15.3 \pm 0.71$  liters O<sub>2</sub> per kg.<sup>3</sup>. Practically the same result was observed with the control animals namely  $15.4 \pm 0.23$  liters O<sub>2</sub> per kg.<sup>3</sup>. In the averages for the phosphorus-deficient animals one result with animal 27 has been omitted since an R. Q. of only 0.62 showed that it was erroneous. The conclusion that phosphorus deficiency did not materially affect the O<sub>2</sub> consumption during fasting is, however, justified, whether or not this erroneous result is included in the calculation. It is interesting to note that animal 27 did not decrease its O<sub>2</sub> consumption when the food intake was reduced from the one-half maintenance level to fasting; it showed even a slight increase from 16.1 liters O<sub>2</sub> per day per kg.<sup>3</sup> at one-half maintenance to 16.8 liters O<sub>2</sub> per day per kg.<sup>3</sup> during fasting which, however, is statistically not significant.

Assuming a linear relation between O<sub>2</sub> consumption per kg.<sup>3</sup> and food intake per kg.<sup>3</sup> in order to give an approximate summary of our results (as explained for CO<sub>2</sub>) the following regression equations have been calculated by the method of least squares applied to the data obtained in respiration trials 13 to 25: for phosphorus-deficient animals

$$O = 14.89 + 0.108 f$$

For the control animals

$$O = 15.04 + 0.067 f$$

The terms in these equations are defined as follows:  $O$  = liters O<sub>2</sub> consumed per day per kg.<sup>3</sup>.  $f$  = grams dry matter of food eaten per day per kg.<sup>3</sup>.

The regression coefficients for O<sub>2</sub> consumption on food intake (0.108 and 0.067) are considerably lower than the regression coefficient for CO<sub>2</sub> production on food consumption (0.248 and 0.233). This result is to be expected if the food

contains mainly carbohydrates. If the animal has an R. Q. of 0.7 during fasting and subsequently raises the R. Q. to 1.0 by ingestion of carbohydrates, then, theoretically, the  $\text{CO}_2$  production may increase 40% without a rise in the  $\text{O}_2$  consumption.

The regression coefficient of  $\text{O}_2$  consumption on food consumption is higher in the phosphorus-deficient animals than in the controls. This relation parallels that for  $\text{CO}_2$ . The difference is likewise not statistically significant. The probability that the difference is a matter of random is 20 to 30%. At least forty-nine additional pair trials with the same results as those discussed here would be necessary to prove with statistical significance the increase in the stimulating effect of food consumption on  $\text{O}_2$  consumption as a result of phosphorus deficiency.

*R. Q.* During the second and third periods the R. Q. of the phosphorus-deficient animals varied from 0.708 to 1.013 (maximum food intake) (table 7). The corresponding range for the control animals was from 0.709 to 1.042.

The R. Q. of both groups of animals is essentially influenced by the plane of nutrition. The correlation coefficient of R. Q. and the food intake per  $\text{kg.}^3$  amount to 0.96 for the phosphorus-deficient and to 0.98 for the control animals.

The linear regression equations calculated by the method of least squares are as follows:

For the phosphorus deficient animals

$$R. Q. = 0.731 + 0.10 f$$

For the control animals

$$R. Q. = 0.730 + 0.009 f$$

The term  $f$  stands, as in the previous equations, for grams dry matter of food consumed daily per  $\text{kg.}^3$ .

During the fourth day of fasting the average R. Q. of the phosphorus-deficient heifers amounted to  $0.715 \pm 0.009$  (three trials); the R. Q. of the control animals to  $0.716 \pm 0.004$  (four trials).

The results of the respiration trials during the second and third periods of the experiment lead thus to the conclusion



that phosphorus deficiency had no influence on the R.Q. of the fasting animals. The slight increase in the R.Q. of the phosphorus-deficient animals with food over that of the control animals is insignificant.

*Phosphorus deficiency and fasting metabolism.* The caloric value of  $\text{CO}_2$  produced by our beef heifers during the fourth and the fifth day of fasting amounted to 6.52 kg. calories per liter. This result has been obtained by calculating the energy loss on the basis of the C and N balances in

TABLE 8  
*Heat increment and partial energy efficiency. Example of calculation*

ANIMAL NO.	TRIAL NO.	FOOD ENERGY METABOLIZABLE PER DAY PER kg. $\frac{3}{4}$	HEAT PRODUCED PER DAY PER kg. $\frac{3}{4}$	INCREASE IN METABOLIZABLE FOOD ENERGY $\Delta U$	INCREASE IN HEAT PRODUCTION $\Delta Q$	RELATIVE HEAT INCREMENT $\frac{\Delta Q}{\Delta U}$	PARTIAL ENERGY EFFICIENCY $1 - \frac{\Delta Q}{\Delta U}$
<i>Phosphorus deficient</i>		kg. cal.	kg. cal.	kg. cal.	kg. cal.	%	%
27	17	0.0	67.8				
	15	40.3	81.4	40.3	13.6	33.7	66.3
	13	73.7	88.5	33.4	7.1	21.3	78.7
	24	0.0	77.1	35.8	— 1.2	— 3.4	103.4
	22	35.8	75.9	15.0	5.2	34.7	65.3
	20	50.8	81.1				

trials 24 and 25. If this figure is used in order to calculate the heat production of the animals during the fifth day of fasting in the second and third periods, one derives an average daily fasting 'katabolism per kg.'<sup>3</sup> of  $(69.3 \pm 3.0)$  kg. calories for the phosphorus-deficient animals (four trials) and  $(70.3 \pm 5.0)$  kg. calories for the controls (three trials).

*Calorigenic action of the food and partial energy efficiency.* Table 8 gives an example of the calculation of the relative heat increment, that is, the heat increment or calorigenic action of the food in per cent of the metabolizable energy. The table also shows how the partial-energy efficiency is derived from the relative heat increment.

Fifteen out of twenty comparisons between different food levels in our trials confirm earlier observations of Forbes ('28) and Mitchell and co-workers ('32) that the calorogenic action per unit of food energy in cattle is increased with increasing plane of nutrition.

Thus in eight comparisons of heat production of our heifers at normal phosphorus intake there was an average calorogenic action of  $11.8 \pm 3.4\%$  of metabolizable food energy for the change from fasting to one-half maintenance, while the corresponding calorogenic action in changing from one-half maintenance to full maintenance amounted to  $26.6 \pm 3.9\%$ .

Since the partial efficiency is thus affected by the plane of nutrition the comparison between the efficiency of phosphorus-deficient and control animals should be made at the same food level.

Two animals are assumed to be at the same food level if they take in equal amounts of metabolizable energy of a given food mixture per kg.<sup>3</sup> (p. 128). The results of two heifers have been calculated to an intake of 70 kg. calories metabolizable food energy per kg.<sup>3</sup>. The method used is illustrated in table 9.

The results of these calculations are summarized in table 10. The partial efficiency thus derived from our results is high throughout compared with the efficiency for fattening which would be expected on the basis of the composition of the food. Using Kellner's figures (Kellner, '19, appendix, table 1) one may calculate for example that the 'Wertigkeit' (value) of ration 1 was 82.7%, that of ration 5, 86.5%. Kellner's further data (Kellner and Köhler, '00) for the metabolizable energy in digestible protein (4.96 kilocalories per gram) digestible fat (8.82 kilocalories per gram) digestible crude fiber (3.65 kilocalories per gram) and starch (3.76 kilocalories per gram) together with the partial efficiency of the metabolizable energy for fattening (namely, 45% for protein, 56% for fat, 63% for fiber and 59% for starch), lead to the calculation of a partial efficiency of 46.4% of the metabolizable energy for our ration 1 and 50.7% for ration 5.

The 'Wertigkeit' mentioned above has been used for this calculation.

If the net energy for these two rations is calculated according to Armsby's method (Armsby, '22, p. 674) a partial efficiency of 61% of the metabolizable energy results for ration 1 and 64% for ration 5. The fact that Armsby's values are higher than those of Kellner and our actual results still higher than the values obtained from Armsby's calculation is not surprising. Kellner's data are strictly fattening values for

TABLE 9

*Calculation of partial efficiency at a food level of 70 kg. cal. metabolizable energy per kg.<sup>2</sup>*

*Animal 27*

Heat production at an intake of 73.7 kg. cal. metabolizable food energy per kg. <sup>2</sup> (trial 13)	88.5 kg. cal. per kg. <sup>2</sup>
Heat increment per calorie metabolizable energy between an intake of 40.3 kg. cal. (trial 15) and 73.7 kg. cal. per kg. <sup>2</sup> (trial 13): 0.21 kg. cal.	
Heat increment for a difference of 3.7 kg. cal. metabolizable energy	0.8 kg. cal. per kg. <sup>2</sup>
Interpolated heat production at an intake of 70 kg. cal. per kg. <sup>2</sup>	87.7 kg. cal. per kg. <sup>2</sup>
Heat production during fasting (trial 17)	67.8 kg. cal. per kg. <sup>2</sup>
Calculated heat increment for increase of food energy from fasting to 70 kg. cal. per kg. <sup>2</sup>	19.9 kg. cal. per kg. <sup>2</sup>
Heat increment in per cent of increase in metabolizable food energy: $\frac{19.9}{70} \times 100 =$	28.5%
Partial energy efficiency between fasting and intake of 70 kg. cal. metabolizable food energy: $100 - 28.5 =$	71.5%

adult steers, Armsby's method is based on experiments at supermaintenance as well as submaintenance levels with steers ranging from 9 to 60 months of age (Armsby and Fries, '15, p. 438) and the partial efficiency in our trials was measured at submaintenance of young animals.

Kellner's as well as Armsby's method of calculating net energy leads to the conclusion that the partial efficiency of food B (particularly ration 5) should be higher than that of food A (particularly ration 1). Our data presented in table 10 are in agreement with this prediction.

During the preliminary period, when both heifers received dicalcium phosphate as a supplement to their phosphorus-deficient diet, the partial-energy efficiency was practically the same. During the second period of our trial, when animal 27 was changed to the phosphorus-low diet and developed symptoms of phosphorus deficiency, its partial-energy efficiency was decreased, while its mate that was still receiving dicalcium phosphate utilized the food energy with the same partial efficiency as in the preliminary period.

TABLE 10

*Partial energy efficiency of phosphorus-deficient and control heifers. Calculated to a food level of 70 cal. metabolizable food energy per kg.<sup>3</sup>*

	ANIMAL 27		ANIMAL 29	NO. 32		NO. 18
	Phosphorus deficient	Normal phosphorus intake	Normal phosphorus intake	Phosphorus deficient	Normal phosphorus intake	Normal phosphorus intake
	%	%	%	%	%	%
Food A rations (1 to 4)						
Preliminary period trials 2, 4 and 11		83.1	81.4		77.3	73.3
Second period of phosphorus deficiency trials 13, 15, 17	71.5		83.3	69.6		
Food B (rations 5 and 6)						
Third period of phosphorus deficiency trials 20, 22, 24	84.7		88.7			78.0

The partial efficiency of the phosphorus-deficient animal during the third period is again lower than that of its pair mate on the same diet supplemented with phosphate.

The partial efficiency of the Angus heifer, 32, at the start of the experiment was slightly below that of the two Hereford heifers. Phosphorus deficiency in this heifer also lowered the partial-energy efficiency, namely, from 77.3 to 69.6% during the second period of our experiment, thus confirming the observation with the Hereford heifer, 27. During the third period of the experiment heifer 32 had unfortunately lost its appetite to such a degree that it would not eat more than a one-half maintenance ration.

The decrease in partial-energy efficiency goes parallel with the decrease in the efficiency of protein utilization. The increase in the katabolism of protein in the phosphorus-deficient animals alone cannot, however, account for the entire increase in the calorogenic action of the phosphorus-deficient food.

## 5. SUMMARY

Two beef heifers fed a phosphorus-deficient diet containing only 0.13% of phosphorus ceased to grow after 6 months of the low-phosphorus regimen, maintained their body weight during the next year (with 0.09% phosphorus in the food), and finally when fed a diet still lower in phosphorus (with 0.068% phosphorus) lost weight. Two control heifers fed with the same food but supplemented with dicalcium phosphate so that the phosphorus content was above 0.4% increased their weight while their phosphorus-deficient pair mates ceased to grow.

The phosphorus-deficient animals developed bone-chewing and coprophagia. The inorganic phosphorus content in their blood dropped from 9.0 to 3.9 mg. of phosphorus per 100 cc. of blood serum while that of the controls remained at the initial level of about 9 mg. phosphorus per 100 cc. of serum.

Phosphorus deficiency had no effect on body temperature, digestibility and metabolizability of the food energy, R. Q. and fasting katabolism.

Phosphorus deficiency increased slightly the regression of  $\text{CO}_2$  production and  $\text{O}_2$  consumption on food intake (not proved with statistical significance). It decreased the partial efficiency of energy utilization, the efficiency of food protein for sparing body protein, and the appetite of the animals.

Phosphorus deficiency lowers the total efficiency of energy utilization ( $\frac{\text{Energy in the product}}{\text{Total food energy}} = \text{mean partial efficiency}$  —  $\frac{\text{Total intake of food energy}}{\text{Fasting katabolism}}$ ) mainly by lowering the appetite and secondly by lowering the partial efficiency whereas it does not seem to influence the fasting katabolism.

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# THE ACTIVITY OF YEAST EXTRACT IN THE PREVENTION OF RENAL HYPERTROPHY CAUSED BY HIGH PROTEIN DIETS <sup>1</sup>

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## ONE FIGURE

(Received for publication April 28, 1936)

In a previous publication Longwell, Hill and Lewis ('32) reported that excessive amounts of protein in the diet of the white rat resulted in renal hypertrophy, an effect which could be prevented by the addition of sufficient amounts of yeast extract to the diet. These results were in agreement with those of Reader and Drummond ('26) and of Hartwell ('28), but in disagreement with the results of Francis, Smith and Moise ('31), a fact which was overlooked when the original paper was presented for publication. The evidence obtained by MacKay ('33) indicates that vitamin B (old terminology) is antagonistic to this action of casein. The earlier conclusions were drawn from a small part of a larger study concerned with the effect of cystine upon the kidney. In view of the disagreement between that study and the results of Francis, Smith and Moise, additional experiments concerning the effect of yeast extract on the enlargement of the kidney caused by casein are herein reported.

<sup>1</sup> Presented in part before the Division of Biological Chemistry at the eighty-sixth meeting of the American Chemical Society, September 11, 1933.

## METHODS

The experimental procedure was the same as that formerly described (Longwell, Hill and Lewis, '32) with a few additions and modifications. Vitamin-free casein<sup>2</sup> was used as the protein. We formerly made use of alcohol-extracted casein prepared in our own laboratory. In the feeding regime of those animals which received a high protein diet, the casein was substituted for an equicaloric quantity of cornstarch in the Sherman and Spohn ('23) diet.

The undifferentiated yeast extract was made according to the method of Williams and Lewis ('30), and the amount fed daily is expressed in gram yeast equivalents (g.y.e.), the gram yeast equivalent being that amount of yeast extract obtained from 1 gm. of dry yeast. In the experiments in which the separated vitamins of the water-soluble B group were used, autoclaved liver supplied a source of vitamin G, and the vitamin B was fed in the form of tikitiki<sup>3</sup> extract (Evans and Lepkovsky, '30).

The experimental animals, male albino rats, were fed for a period of 21 days, beginning when they were 28 days old. They were killed by a head blow and the kidneys and hearts were weighed in glass-stoppered weighing bottles after the large blood vessels had been carefully removed from these organs. The kidney weights obtained are compared to the body weight and to the heart weight as a means of measuring renal hypertrophy. The measurements are expressed as the K:B (kidney:body weight) ratio and the H:K (heart: kidney) ratio, respectively. In our earlier study we used only the K:B ratio as a means of measuring renal hypertrophy. This method is possibly open to criticism because of the complicating factor of inanition to which the animals which received the restricted vitamin supplements were subject. Francis, Smith and Moise ('31) used the heart: kidney

<sup>2</sup> Obtained from The Casein Manufacturing Corporation of America.

<sup>3</sup> The yeast used in this investigation was furnished by Standard Brands, Inc., and the rice polishings were furnished by the Louisiana State Rice Milling Company. The cooperation of these companies is gratefully acknowledged.

ratio for the measurement of renal hypertrophy. This method rules out any possible effect of inanition provided the assumption is correct that there are no changes in the circulatory system (heart) as a result of the ingestion of abnormally great amounts of protein or abnormally small amounts of vitamin.

In a further attempt to rule out the factor of inanition, a comparison was made between the kidney weight and the fat-free body weight. It was thought that the fat-free body weight might be used in this manner because of the loss of body fat which occurs as the result of inanition from whatever cause. After removal of the kidneys and heart, the carcass of the animal was heated on a water bath in a strong solution of potassium hydroxide until it had dissolved completely. The solution was cooled, acidified with sulfuric acid, and extracted repeatedly in tall cylinders with petroleum ether. The extraction was accomplished by shaking the hydrolyzed carcass with ether and allowing the ether extract to separate, after which it was pipetted off and evaporated to a constant weight in a tared flask.

#### RESULTS

Tables 1 and 2 show the results of these experiments. They confirm our original contention that sufficient yeast extract as a dietary supplement inhibits the renal hypertrophy which occurs as a consequence of the ingestion of excessive amounts of casein. When the higher amounts of protein were fed (80% of the diet, table 2), the administration of large amounts of yeast extract did not completely prevent hypertrophy of the kidneys, but the larger quantities (3.0 and 4.0 g.y.e.) tended to inhibit this effect of casein.

Table 1 contains data on the relationship between the kidney weight and the fat-free body weight. The results show the same general trend when the extent of renal hypertrophy is measured in this manner as do the results in the same groups when the whole body weight is used as a basis of comparison. Figure 1 depicts the quantitative estimation of

the amount of fat present in the bodies of the rats of groups 1 to 10, inclusive.

Race, Longwell and Lewis ('32) made an attempt to show the effect of inanition on the K:B ratio. Food curtailment was used to retard growth so that the rats demonstrated

TABLE 1

*The effect of yeast extract in the prevention of renal hypertrophy caused by casein*

GROUP	NUMBER OF RATS	YEAST EXTRACT	CASEIN	K: B RATIO <sup>2</sup>	K: B RATIO (FAT-FREE BODY WEIGHT)	H: K RATIO <sup>3</sup>
1	16	<i>g.y.e.</i> <sup>1</sup> 1.0	% 18	0.012	0.012	0.408
2	6	0.0	40	0.019	0.020	0.288
3	6	0.2	40	0.014	0.014	0.343
4	5	0.4	40	0.013	0.014	0.359
5	6	0.6	40	0.013	0.014	0.361
6	6	0.8	40	0.013	0.014	0.375
7	6	1.0	40	0.013	0.014	0.393
8	6	1.5	40	0.012	0.013	0.373
9	2	1.5	80	0.021	0.021	0.326
10	3	2.0	80	0.018	0.019	0.333

<sup>1</sup> One g.y.e. (gram yeast equivalent) is the amount of extract which was obtained from 1 gm. of yeast.

<sup>2</sup> Ratio between the kidney weight and the body weight.

<sup>3</sup> Ratio between the heart weight and the kidney weight.

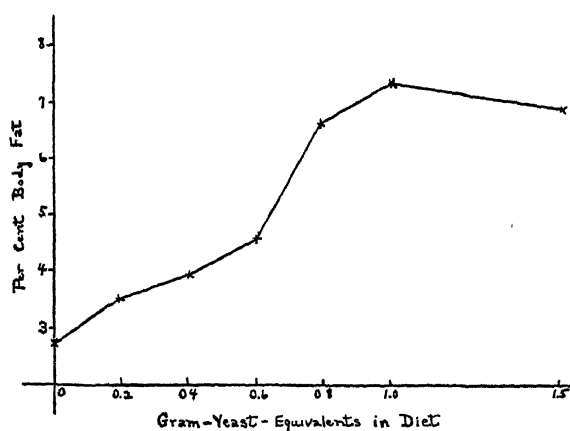


Fig.1 Effect of yeast extract on body fat of rats on 40% protein diet.

approximately the same body weight change as a group deprived of yeast extract. The K:B ratio was 0.011 and indicates that inanition, per se, was not a factor which influenced the results herein reported.

Groups 27 and 28 (table 2) were fed autoclaved liver as a source of vitamin G and tikitiki extract as a source of vitamin B. The only supplement given to group 29 in addition

TABLE 2

*The effect of yeast extract, autoclaved liver and tikitiki extract in the prevention of renal hypertrophy caused by casein*

GROUP	NUMBER OF RATS	YEAST EXTRACT	CASEIN	K : B RATIO	H : K RATIO	AVERAGE WEIGHT GAIN
		<i>g.v.e.</i>	<i>%</i>			<i>gm.</i>
11	8	1.00	20	0.013	0.356	26.4
12	4	0.00	40	0.015	0.306	0.7
13	8	0.25	40	0.015	0.323	7.5
14	8	0.50	40	0.014	0.278	13.6
15	8	1.50	40	0.012	0.386	29.8
16	8	0.00	60	0.018	0.269	-2.9
17	8	0.25	60	0.017	0.295	0.5
18	8	0.50	60	0.017	0.215	8.4
19	8	1.50	60	0.017	0.318	31.7
20	8	3.00	60	0.014	0.341	34.8
21	4	0.00	80	0.027	0.248	-7.2
22	8	0.25	80	0.020	0.265	4.8
23	8	0.50	80	0.018	0.299	6.4
24	8	1.50	80	0.019	0.287	18.3
25	8	3.00	80	0.016	0.341	27.6
26	8	4.00	80	0.017	0.310	23.8
27	8	<sup>1</sup>	20	0.011	0.387	38.3
28	8	<sup>1</sup>	40	0.013	0.371	42.8
29	8	<sup>2</sup>	40	0.015	0.310	26.6
30	8	<sup>3</sup>	40	0.015	0.391	1.3

<sup>1</sup> One gram autoclaved liver and extract from 1 gm. of rice polishings daily.

<sup>2</sup> One gram autoclaved liver daily.

<sup>3</sup> Extract from 1 gm. of rice polishings daily.

to the basal diet was autoclaved liver. Group 30 received tikitiki extract as the only dietary supplement. The results with groups 27 and 28 show substantially the same relationship between the kidney weight and the other body measurements used as that established in the groups which received

yeast extract. There is no difference between the K: B ratio in group 29 (autoclaved liver) and group 30 (tikitiki). These data do not support the contention of Hartwell ('28) that the effective agent of yeast is the heat-stable factor, but are in agreement with the findings of Prunty and Roscoe ('33). We note this fact without undue emphasis because the results were obtained with two small groups only, and because liver and rice polishings were used as a source of the vitamins, a procedure which introduced substances into the dietary regime which were not identical with the factors obtained from yeast.

The data of group 30 (table 2) show that the animals which received tikitiki extract grew much more poorly than did those which received autoclaved liver (group 29). This result was probably due to the growth promoting properties of vitamin G. Furthermore, the growth of these animals was not greatly less than that of the animals which received whole yeast extract. According to Stiebeling and Alleman ('33) extraction with alcohol destroys part of the vitamin G. Since this vitamin is the limiting factor in the growth of the animals under the conditions of this experiment, we feel that the growth rates of the animals which received the undifferentiated vitamin B complex as compared to group 29 are due to partial destruction of vitamin G in the whole yeast extract. In spite of the differences in rates of growth, the results obtained on the weights of the kidneys appear to be significant.

#### DISCUSSION

The workers who have observed that yeast has an inhibitory effect upon renal hypertrophy caused by protein have usually attributed this effect to its vitamin B or G content. It will be recalled that Francis, Smith and Moise and Prunty and Roscoe have reported that whole yeast and the heat stable factor of yeast, respectively, do not inhibit renal hypertrophy. These latter findings are confirmed in this report. When one attempts to explain these negative results in the light of variations in the vitamin preparations, one encounters

the difficulty that no sharp separation has been made between the factors used. It is true that some of the results have been obtained with preparations which, according to the known properties of vitamins B and G, should contain one of these to the exclusion of the other. However, these preparations contain many additional vitamin components and until preparations of definite vitamin content have been studied or until the factors are used in the pure form, the relationship of accessory food substances to the development of the kidneys should not be attributed to any definite substance.

Reader and Drummond ('26) used whole yeast extract. The method of preparation is not described, but the material was active in the prevention of renal hypertrophy when large amounts were used. Hartwell ('28) found that untreated marmite gave positive results. MacKay ('33) utilized an Osborne and Wakeman ('19) concentrate prepared by alcoholic fractionation of a yeast extract made with very dilute acetic acid, and found it to be active. Our own results were obtained with an alcoholic extract of yeast. The methods of preparation of these various extracts are not identical, and the final products are probably quantitatively different with respect to vitamins B and G, and possibly they are qualitatively different with respect to the other factors present in yeast. These variations may be brought about by differences in the original yeast, and by destruction or by poor extraction of the dietary factors in the process of preparation. Even though these differences may exist, they do not seem to be significant, as far as the effectiveness of yeast is concerned, since all of the above mentioned workers obtained positive results with undifferentiated extracts.

Marmite which had been autoclaved  $4\frac{1}{2}$  to 5 hours was used effectively by Hartwell ('28) in part of her experiments. Opposed to this evidence stand the findings of Prunty and Roscoe ('33) who found autoclaved acetic acid extract of yeast inactive. Autoclaved liver was inactive in our own experiments. The use of this latter material, however, in all probability introduces factors into the diet which are not identical with those found in yeast. The existing evidence

does not permit allocation of the activity of yeast in the prevention of kidney hypertrophy to vitamin G.

Francis, Smith and Moise ('31) are the only workers who have reported a failure of yeast to counteract the effect of a diet rich in protein on the kidney. There is no evidence in their work that the diet did not contain the requisite accessory factors. The vitamin-containing supplements in question were given in the form of whole, dry yeast fed daily in amounts far in excess of that necessary to insure good growth. It is possible, however, that these factors were quantitatively insufficient to counteract the effects of such excessive levels of dietary protein (compare MacKay, '33). Additional supplements of autoclaved yeast and tikitiki extract were given to some of their animals without any effect, however, and this fact makes it likely that the yeast supply was adequate.

Critical analysis of these various reports forces one to the conclusion that differences in results must have been caused by some factors other than the accessory dietary supplements obtained from yeast. In our own work, groups 9 and 10, table 1, show only five animals. These represent the survivors of nineteen animals which were started on the feeding regime indicated. The only variable factor in these diets was the protein. Protein used at a level of 80% of the diet had a severe toxic effect on these animals. Not only did they fail to grow, but also they began to suffer from extreme inanition soon after the feeding was begun. In spite of the fact that they ate little and failed to consume double the amount of protein taken by the groups which received the 40% protein diet, fourteen of the nineteen rats died before termination of the experimental period of 21 days. No such evidence of intoxication was observed in the rats of groups 21 to 26, table 2 (second series). The diets of the animals of these latter groups were identical with those of groups 9 and 10 of the first series except that we had received a new supply of casein before their feeding was begun. It is possible that some difference in the preparation of the casein is an effective factor in these experiments. This hypothesis is being subjected to investigation.



A second possible explanation of these discrepancies appears upon examination of the data of Reader and Drummond ('26, p. 1261). It will be noted that in their groups C and D the kidneys of the animals of group C were larger than those of group D. The authors do not seem to consider that this difference is significant, but the kidneys of the animals of group C are 31.9% larger by reference to body weight than those of the animals of group D. The diets of these two groups differed only in that diet C contained no fat except that fed in the cod liver oil, whereas diet D contained 18 parts of 'hardened fat' in addition to the cod liver oil. It is of extreme interest that the only other experiments in which the diets contained no fat are those of Francis, Smith and Moise ('31), and their results show yeast to be ineffective as a preventative of renal hypertrophy. The possibility of fat as a key factor in the effectiveness of yeast in the prevention of this type of kidney enlargement is being investigated by us at the present time.

Our animals did not make quite normal growth. One might suppose that systemic growth may be a factor which influences the size of the kidneys. Reference to group 11, table 2, however, in which the protein of the diet was low, shows that there was no renal hypertrophy in spite of the fact that optimum growth was not attained. While one must admit that the growth element may be of influence, we feel that the difference in growth shown by the animals on the low and high protein diets was not sufficiently great to vitiate our conclusions.

#### SUMMARY

Young albino rats fed diets containing amounts of casein in excess of that necessary for optimum growth developed renal hypertrophy. This tendency was prevented or inhibited by yeast extract supplements. Neither tikitiki extract nor autoclaved liver prevented entirely the renal hypertrophy which resulted from a 40% protein diet, but they had an inhibitory effect when given together. The results were not due to inanition.

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# A STUDY OF THE FECAL FLORA AND THE LINE TEST OF NORMAL RATS, RACHITIC RATS AND HEALING RACHITIC RATS <sup>1</sup>

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THREE FIGURES

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## I. INTRODUCTION

### *A. Foreword*

Although the line test is the official method for determining the extent of rickets or healing in the excised joint of an animal's leg, investigators (Jephcott and Bacharach, '26 and Grayzell and Miller, '28) have attempted to use the change in the pH of the feces as an indication of rickets or healing in the animal. This procedure when applied to children proved impracticable (Redman, '28).

Hess and Torrey ('32) tried to correlate the changes in the bacterial flora with the changes in pH by experimenting with the intestinal flora of rachitic rats and rats which were irradiated with ultraviolet rays.

### *B. Object of the experiment*

The object of this investigation was to compare the pH and the bacterial flora of the feces of normal rats, rachitic rats and rats which were healed of rickets by feeding irradiated ergosterol and vitamin D milk.

<sup>1</sup> This paper was awarded a prize by the American Association of Medical Milk Commissions, Inc. and Certified Milk Producer's Association of America, Inc., under the auspices of the Milk Commission of the Medical Society of the County of New York.

## II. EXPERIMENTAL

*A. Feeding procedure*

Twenty-eight- to 29-day-old albino rats weighing 48 to 50 gm. each were used. All rats had distilled water and Steenbock no. 2965 ration ad lib throughout the experimental period of 29 days. In addition to the basal diet, the following groups were fed special sources of vitamin D from the nineteenth through the twenty-ninth day (vitamin D testing period), the correct degree of rickets having been shown (line test) (Medical Research Council, '32) by the six test rats killed on the nineteenth day.

## 1. Negative controls

a. Seventeen rats—nothing added to the basal diet.

## b. Milk controls

(1) Five rats—6.0 cc. of plain certified milk fed to each rat.

(2) Five rats—6.4 cc. of plain pasteurized A milk fed to each rat.

## 2. Healing rats

a. Ten rats—irradiated ergosterol containing 2.7 U. S. P. X. vitamin D units was fed to each rat.

## b. Milk-fed rats

(1) Five rats—6.0 cc. of vitamin D certified milk containing 2.7 U. S. P. X. units was fed each rat.

(2) Five rats—6.4 cc. of vitamin D pasteurized A milk containing 2.7 U. S. P. X. vitamin D units was fed to each rat

## 3. Normal rats

Eighteen rats used: Irradiated ergosterol containing 5.4 U. S. P. X. vitamin D units was fed to each rat every 4 days from the first day of the experiment throughout the 29 days.

*B. Bacteriological methods*

The routine bacteriological examination of the stools was carried out as recommended by Torrey ('26) and Hess and

Torrey ('32). Brom cresol purple lactose agar and blood agar plates were seeded with the stool suspensions (4 mm. loop = approximately 0.01 cc. was used) to determine the number of acid producing and non-acid producing organisms and green streptococci, hemolytic streptococci and coli respectively.

Plain beef agar plates were used to determine the bacterial count. All of the agar plates were read after 48 hours at 37°C.

The proteolysis and the quantity of gas produced were determined by inoculating cooked meat media covered with vaseline with the original suspension. Two tubes were inoculated with each fecal sample, one heated to 80°C. for 10 minutes to exclude all vegetative organisms. Both tubes were incubated for 4 days at 37°C. The proteolysis was measured by the amount of digestion taking place in the cooked meat media and the quantity of gas was measured by the height of the vaseline in the tube.

The pH of these fecal suspensions was determined by means of the Clark and Lubs color indicators (Clark, '25). The 1-10 dilution of fecal suspension was used with which to test the pH and this was compared with known standard buffer solutions.

From the brom creosol purple lactose plates, fishings were made and the organisms identified by subsequent cultures on milk medium, blood agar plates, lactose and dextrose broth.

### *C. Results*

1. *pH results.* The range in pH of the feces of the normal rats was 6.20 to 6.60 throughout the entire 29-day period. Figure 1 shows the average pH of stool samples taken on the same day. The animals which gave these acid pH results showed a normal line test (no rickets) (Medical Research Council, '32).

The rachitic rats which were fed the Steenbock ration only had an acid pH (6.40 to 6.80) at the beginning of the test but as the rickets in the rat progressed, the pH became more basic (up to 7.60). The line test in these cases revealed

excellent rickets or moderate rickets. The average pH of these rats which became rachitic is shown clearly in figure 1. The rats fed the plain pasteurized and plain certified milk gave parallel results.

The pH of the feces of the healing rats on the other hand, was basic at the end of the nineteenth day but became more acid after the irradiated ergosterol containing vitamin D was fed. The average pH at the beginning of the healing period (nineteenth day) was 7.63 and at the end of the period

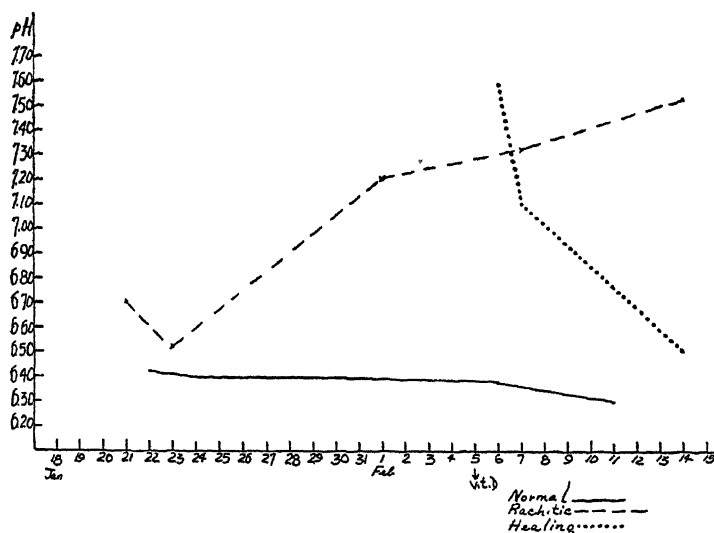


Fig. 1 Curves indicating the change in pH in the feces of normal, rachitic and healing rats. (Average pH.)

(twenty-ninth day) it was 6.53, almost hitting the normal curve. The line test checked these results with a 2 + healing. The rats fed the vitamin D milk gave almost identical results.

2. *Bacteriological results.* Acid-producing colonies were predominant on the brom cresol purple lactose agar plates seeded with the fecal suspensions of the normal rats. This was apparent from every sample taken during the test period. As the rats became rachitic however, the number of acid-producing colonies grew smaller and the number of colonies producing no change in the indicator increased. The healing

rats showed a dominance of the latter colonies at the beginning of the vitamin D period but at the end of that period, whether the rats were fed the irradiated ergosterol or the vitamin D milk the plates showed an increase in acid-producing colonies almost consistently. The averages of these two types of colonies throughout the experiment are shown in figures 2 and 3.

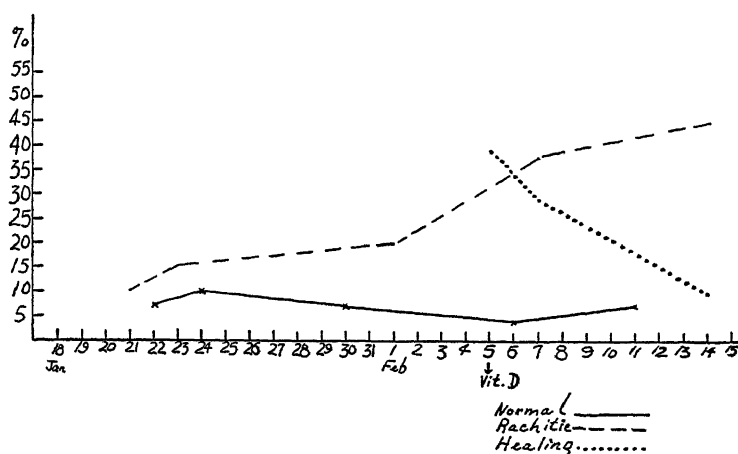


Fig. 2 Curves indicating the percentage of non-acid producing colonies from the feces of normal, rachitic and healing rats. (Average percentage.)

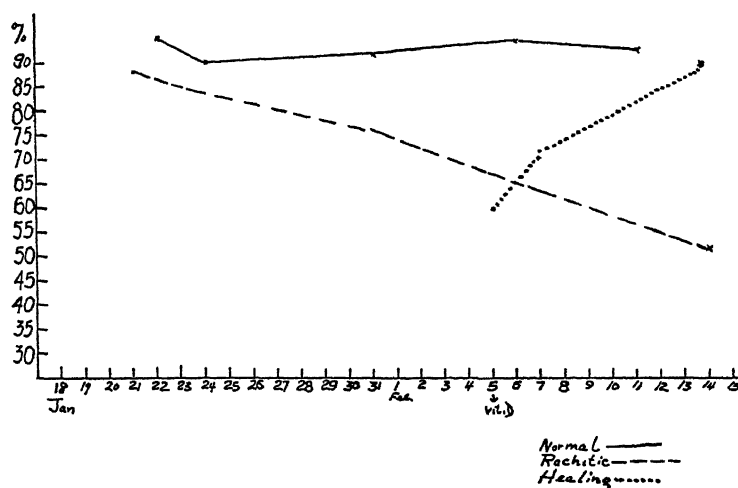


Fig. 3 Curves indicating the percentage of acid-producing colonies from the feces of normal, rachitic and healing rats. (Average percentage.)

The number of bacteria per cubic centimeter showed little difference between the feces of the various groups of rats tested.

The character of the bacterial flora recovered from the plates varied in the different groups. The abundance of the colon bacillus was observed to be definitely less on the plates from the samples taken at the end of the rachitic period than at the end of either the normal or healing periods. This was true also for the plain milks as compared with the vitamin D milks. There were slightly more streptococci for the most part green, from the rachitic rats and those fed the plain milks than from the normal, the rats healed with irradiated ergosterol or those fed the vitamin D milks.

There was no difference between the groups as to proteolytic action but there was a slight decrease in gas formation from the stool specimens of the rats on the plain Steenbock ration and those on the Steenbock ration plus the plain milk.

### III. DISCUSSION

The pH followed the same trend as reported by other investigators. The interesting fact observed, however, was the almost direct correlation of the acid producing colonies on the lactose agar plates with the pH of the same samples. The pictures of the curves in figures 1 and 3 are practically identical, showing that the change in pH coincides accurately with the acid-producing colonies on the plates.

The number of bacteria per cubic centimeter in each fecal specimen reported from the pour plates varied to a certain extent but in no definite direction. The possibility of percentage error in diluting and plating is too large to permit of definite statements as to any differences.

The most pronounced feature in the fecal flora was the decrease of *B. coli* in the feces from the rachitic rats and their subsequent increase as the rats were being healed. The number of *B. coli* in the feces of the normal rats was much greater than in the feces of the rachitic rats. The average taken at the end of the experimental period of the percentage of *B. coli* showed the following:



Normal rats	15 % <i>B. coli</i> in the fecal flora
Rachitic rats	0.3% <i>B. coli</i> in the fecal flora
Healing rats	20 % <i>B. coli</i> in the fecal flora

There was also a slight increase in the green streptococci from the rachitic rat stools which was not apparent on the blood plates from the normal rats or the rats healed with irradiated ergosterol or vitamin D milk.

The pH according to the experimental results cited can be used roughly as a qualitative indication of the presence or the lack of vitamin D, provided the diet is known. It is doubted whether at the present time, it can be used alone without the aid of the line test because there is fluctuation from day to day among the individual rats as to their fecal pH during the experimental period and the time response of the different rats to the experimental diets is not sufficiently uniform.

#### IV. CONCLUSIONS

1. The pH reaction of the feces of normal, rachitic and healing rats indicated the type of organisms to be found in the feces, i.e., when the pH was acid (normal or healing rats) the colonies on the plate were acid producing and when the pH reaction was basic (rachitic rats) the colonies on the plate were non-acid forming.

2. A decrease of *B. coli* was evident from the stool samples of the rachitic rats as compared to the samples taken from either the normal or the healing rats.

3. Vitamin D in the pasteurized and certified milk and vitamin D in the irradiated ergosterol effected similar changes in the feces of rats.

4. Provided the diet is known, the tendency toward rickets or healing in rachitic rats can be gauged roughly by means of the basic or acid pH of the feces. However, the extent of the rickets and of the healing cannot be ascertained without the aid of the line test or x-ray.

## ACKNOWLEDGMENTS

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# STUDIES ON THE BLOOD AND TISSUES IN NUTRITIONAL MUSCULAR DYSTROPHY

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TWO FIGURES

(Received for publication May 16, 1936)

## STUDIES ON THE BLOOD

Muscular dystrophy was experimentally produced in rabbits by means of the diet 13 of Goettsch and Pappenheimer ('31). As we reported elsewhere (Morgulis and Spencer, '36 a) recovery from dystrophy which in the course of time leads to complete regeneration of the diseased muscles can be effected by the addition of proper foodstuffs to the basic diet. Using both dystrophic rabbits and those recovering from this disability, we made a systematic study of the blood in the hope that these findings would shed some light on the problem of the causation of this disease.

## METHODS

Blood was obtained from the marginal ear vein, xylol being used when necessary to cause local hyperemia. Sugar determinations were made on blood filtrates prepared according to the Somogyi procedure ('30) and the titrations carried out by the Shaffer-Somogyi method ('33). For the sugar tolerance tests the usual dosage of 3 gm. glucose per kilogram of body weight were administered by stomach tube to rabbits fasting 24 hours. Lactic acid was determined by Wendel's method ('33). The phosphorus determinations were all made by the Kuttner method (Kuttner and Cohen, '27; Kuttner

and Lichtenstein, '30) following the system of analyses outlined by Kuttner and Lichtenstein ('32). For the determination of the acid soluble-P 2 cc. of freshly drawn whole blood were immediately mixed with 18 cc. of 7% trichloroacetic acid to precipitate the proteins. The filtrate represented a 1:10 dilution of the original blood. The adenosinetriphosphate (ATP) content was calculated by multiplying the difference between the P after hydrolysing for 7 minutes at 100° with  $\text{NH}_2\text{SO}_4$  and the inorganic-P of the filtrate by 3/2. The lipid-P and cholesterol were determined in the alcohol-ether extract of the blood prepared according to Bloor ('18). The cholesterol was determined colorimetrically by the acetic anhydride reaction of Liebermann-Burchard.

#### THE GLYCEMIC CURVE

For several years, studying clinical cases of myopathy, we noted in patients with myotonic dystrophy a peculiar and characteristic zig-zag glycemic curve. The sugar tests were carried out by the usual routine following the administration of a standard quantity of glucose to the patients. The shape of the curve, which may be interpreted as manifesting an endocrine disbalance, is not accidental and repeats itself in the same individual with remarkable consistency. We had an opportunity to perform five tests on the same person over a period of 9 months, both before and after treatment with cortin, and except for minor deviations the different curves could be superimposed upon each other. In general, after glucose administration, the glycemic level does not rise as high as in normal individuals, and in some patients the rise is quite insignificant. The peak of the curve is reached in from 30 to 60 minutes, and is followed by a sharp sudden drop, sometimes 15 to 35 mg.% below the fasting level, to rise once more, though less steeply than previously. The curve then slopes off again. In a few instances, where the urinary sugar excretion was also determined, a marked glycosuria (up to 450 mg. glucose) was found at the end of 60 minutes.

In view of this experience with clinical cases of muscular dystrophy we deemed it worth while to study the sugar curve in rabbits suffering from nutritional muscular dystrophy. The average blood sugar value in eight control rabbits was found to be 81 mg.% (63 to 105 mg.%) and in eighteen dystrophic rabbits 78 mg.% (57 to 119 mg.%), showing practically no essential difference between the two groups of

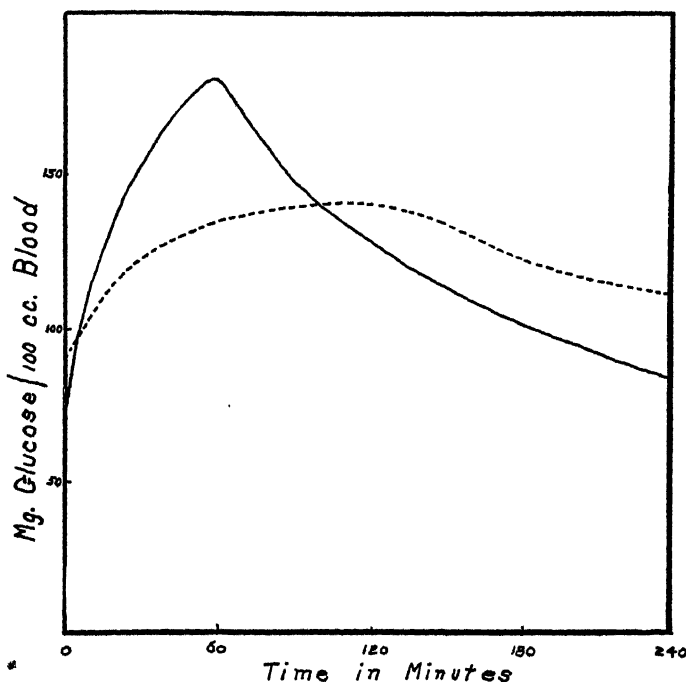


Fig. 1 Glycemic curve of normal rabbits (continuous line) and of dystrophic rabbits (broken line). These are composite curves.

animals. In figure 1 we reproduce the sugar tolerance curves obtained with normal and dystrophic rabbits. These are not individual but composite curves representing the general course of the glycemic reaction. It is obvious that the glycemic curve obtained with rabbits afflicted with muscular dystrophy of nutritional origin is entirely different from the curve prevailing among the human patients with myotonic

dystrophy. Of course, this points to the fact that these two conditions are probably totally different entities, although the histological study of the affected muscles in both instances indicates a remarkable degree of similarity in their microscopic appearance.

The glycemic curve of the dystrophic rabbits varies, however, from the normal curve in that its rise is less steep and slower, the peak being reached in 2 instead of in 1 hour, as in the controls, and the decline is likewise decidedly less sharp than in the latter. The curve of the dystrophic rabbits does not rise as high as that of the normal rabbits. The idea that the peculiar glycemic curve of the dystrophic patients reflects an endocrine disbalance does not apparently gain support from the rabbits with nutritional dystrophy. However, this does not exclude the possibility of endocrine involvement. We have shown elsewhere (Morgulis and Spencer, '36 b) that the adrenal glands and the gonads are considerably reduced in size in the dystrophic animals.

#### LACTIC ACID

Since lactic acid plays such an important role in muscle metabolism we thought it desirable to investigate the behavior of the blood lactic acid in our animals. In six control rabbits we found an average value of 16.6 mg.% (12.6 to 22.4 mg.%) while in eight dystrophic animals the average value was 16.3 mg.% (11.9 to 20.0 mg.%). These findings showing that the lactic acid level, just as we have seen for the blood sugar level, is the same in the control and dystrophic rabbits lead to the conclusion that neither of these constituents is associated with the factors responsible for the production of dystrophy.

#### ACID SOLUBLE PHOSPHORUS COMPOUNDS

Continuing the investigation of substances which play an active part in muscle metabolism, we determined quantitatively the various phosphorus fractions in the blood of our rabbits. The results of these determinations (on five normal and fourteen dystrophic rabbits) are tabulated below.

Since the acid soluble phosphorus compounds are found only in the red blood cells, the analytical results for the various fractions determined have been recalculated on the basis of the red cell count and corrected for 5.7 million cells per cubic millimeter, which was found to be the average for the control rabbits. A comparison, on this basis, shows that the distribution of the acid-soluble-P fractions is practically identical in the bloods from both normal and dystrophic rabbits, as can be seen from this summary of the results:

<i>Phosphorus fraction</i>	<i>Normal</i>	<i>Dystrophic</i>
Inorganic-P	5.2	5.9
Adenosinetriphosphate-P (ATP)	7.2	8.0
Ester-P	36.5	36.1
Total acid-soluble-P	40.7	41.8
Lipid-P	9.2	12.3
Total-P	52.6	60.2

It can be seen from these results that the total blood P is increased in the dystrophic rabbits, this being apparently due largely to the increase in the lipid-P fraction, but that the other P fractions are practically unaffected by the dystrophy. Not only does the lipid-P content show a decided increase but the cholesterol is likewise very much greater in the dystrophic than in the control rabbits. The marked changes in the alcohol-ether extractives are considered in detail in the next section.

#### LIPID PHOSPHORUS AND CHOLESTEROL

Since our study of the blood from animals with muscular dystrophy disclosed significant changes only in the lipid-P and cholesterol, we subjected these blood constituents to a more extensive investigation.

A study made on fourteen normal rabbits shows that the average for cholesterol is 115 mg.% and that for lipid-P 9.2 mg.%. In contrast to these normal values, analyses made on nineteen dystrophic rabbits give an average of 237 mg.% for cholesterol and 13.5 mg.% for the lipid-P content (table 1). This represents an increase in the cholesterol concentration of 107% and in the lipid-P of 45%.

The increase in the cholesterol of the blood apparently runs parallel to the degree of dystrophy, and while the lipid-P likewise seems to bear a relationship to the extent of the dystrophic change, this is not nearly as definite as in the case of the cholesterol. Using the number of plus signs as an arbitrary scale for expressing the degree of dystrophic change in the skeletal muscles, as determined by the microscopic examination of biopsy material, this relationship is

TABLE 1  
*Blood cholesterol and lipid-P (milligrams per cent) in dystrophic rabbits*

RABBIT	FROM CRITICAL POINT <sup>2</sup>		CHOLESTEROL	LIPID-P
	Number of days	Loss of weight in grams		
254	3	90	293	12.5
256	9	400	292	14.7
258	4	140	279	15.4
260 (+++) <sup>1</sup>	12	330	260	12.5
264	4	130	242	12.8
266	3	230	222	10.8
274 (+)	6	270	205	12.4
275 (++)	12	420	233	13.0
279	14	420		13.8
280	6	140	213	12.8
282	4	70	216	12.6
286 (++++)	3	230	293	14.3
296	5	70	242	12.5
303	6	80	184	14.0
306	4	280	235	
313	7	390	286	15.6
319	6	180	250	
328	4	30	240	16.6
329	6	50	316	
Average			250	13.5

<sup>1</sup> The number of plus signs serves as a scale for expressing the degree of muscular degeneration judged on the basis of histological examination.

<sup>2</sup> The 'critical point' is a point on the growth curve of the dystrophic rabbit at which the body weight decreases sharply. For several days preceding this, the growth of the animal instead of being continuous begins to waver, losing a little one day and gaining the next. Definite metabolic changes occur at this period which will be discussed in a separate paper. When the sharp and large loss of weight takes place at the 'critical point' death of the animal is imminent unless recovery is brought about by special dietary means.



shown in the case of a few rabbits for which both the chemical and the histological data are available.

<i>Rabbit no.</i>	<i>Biopsy findings</i>	<i>Cholesterol, mg. %</i>	<i>Lipid-P, mg. %</i>
274	+	205	12.4
275	++	233	13.0
260	+++	260	12.5
286	++++	293	14.3

This view that the changes in the blood lipid-P and especially in the cholesterol are proportional to the development of dystrophy in the muscle system is borne out by

TABLE 2

*Blood cholesterol and lipid-P (milligrams per cent) in rabbits which are recovering from definite dystrophy*

RABBIT	ON RECOVERY DIET		CHOLESTEROL	LIPID-P
	Number of days	Gain of weight in grams		
280	91	1070	208	12.4
288	17	300	171	13.0
291	63	1060	182	9.8
294	95	1770	235	10.4
297	104	1220	125	7.7
298	100	910	118	9.3
300	15	90	261	14.1
300	97	1550	143	9.1
303	15	350	133	10.9
303	59	1140	250	12.4
328	9	200	204	13.6
328	13	290	129	11.0
328	60	1050	100	
Average			174	11.1

further studies. In six rabbits approaching dystrophy the average values for cholesterol and lipid-P were 215 mg.% and 10.4 mg.%, respectively. These values are considerably higher than those found in the control animals but less than those in rabbits with manifest dystrophy (table 1). The results obtained with rabbits recovering from dystrophy on our special diets are even more striking, as can be seen from the data in table 2. With the progress of recovery both the cholesterol and the lipid-P of the blood tend to decrease, the

average values in this group, in spite of its heterogeneous make-up, falling between those of the normal and of the dystrophic groups.

In this connection the data reported in table 3 are particularly significant because they were obtained on the same rabbits in the course of development of the dystrophy and bear out in a striking manner the direct relationship between the gradual rise in the cholesterol and in the lipid-P of the blood as the myopathy progresses. We also followed the changes

TABLE 3  
*Blood cholesterol and lipid-P (milligrams per cent) in rabbits during the progressive development of dystrophy*

RABBIT	DAYS ON DIET	CHANGE IN WEIGHT IN GRAMS (AFTER CRITICAL POINT)	CONDITION	CHOLESTEROL	LIPID-P
306	48	— 30	Slightly dystrophic	172	
306	52	+ 40	Slightly dystrophic	161	
306	56	— 200	Dystrophic	178	
306	57	— 280	Severely dystrophic	235	
266	51	— 230	Dystrophic	222	10.8
266	57	— 430	Severely dystrophic	283	11.8

during recovery from the dystrophy. In one rabbit with dystrophy, whose blood cholesterol and lipid-P were 240 mg.% and 16.6 mg.%, respectively, we brought about recovery from the disease. We determined the cholesterol and lipid-P of the blood on the ninth, thirteenth and sixtieth day, when the animal had gained 200, 290 and 1050 gm. in weight, and found that the cholesterol changed to 204, 129 and finally 100 mg.% while the lipid-P decreased to 13.6 and 11.0 mg.%.

#### STUDIES ON TISSUES

In the preceding section we discussed the changes in the blood of rabbits suffering from nutritional muscular dystrophy. Here we shall consider the chemical changes in the tissues, more particularly in the muscles.

## METHODS

*Removal of muscle.* The rabbits were anesthetized with sodium ortal (100 mg. per kilogram body weight by stomach tube), though toward the end of these experiments chloretone (200 mg. per kilogram) was substituted as the anesthetic, because this proved much more satisfactory in securing complete relaxation over a long period. The gastrocnemius muscle was very carefully dissected from the surrounding structures with as little trauma as possible and was frozen in situ by a mixture of solid carbon dioxide with ether or ethyl chloride, according to the technic described by Davenport and Davenport ('28). The muscle was frozen almost instantly without any noticeable contraction.

For the determination of the glycogen in muscle we used the Good, Kramer and Somogyi ('33) modification of the well-known Pflüger procedure and analyzed the sugar in aliquots, after preliminary hydrolysis, by the Shaffer-Somogyi method ('33). The glycogen content of the muscle was expressed as milligrams glucose per 100 gm. fresh tissue.

For the determination of the total acid soluble-P as well as its various fractions 1 to 2 gm. of the sliced frozen muscle were quickly extracted with ice cold 5% trichloroacetic acid, using 10 cc. per gram of tissue. All the phosphorus determinations were carried out according to the Kuttner method (Kuttner and Cohen, '27; Kuttner and Lichtenstein, '30, '32). The inorganic-P was determined in the original trichloroacetic acid filtrate, while the room temperature hydrolysis was made on the filtrate diluted 1 to 10. The pyrophosphate fraction was determined as in the case of the blood. The hexosemonophosphate-P was determined by the procedure of Cori and Cori ('31-'32).

The total creatinine determination was usually made on muscle which was frozen in situ. The early analyses were made by the method described by Rose, Helmer and Chanutin ('27) which requires about a gram of tissue. Subsequently we followed the micro-procedure of Ochoa and Valdecasas ('29) using about 100 mg. of tissue instead. We also found

that the trichloroacetic acid filtrate prepared for the P determinations could be used satisfactorily in the Ochoa creatinine determination, developing the color with 5% NaOH and matching against a 0.1 mg. standard.

For the lipid-P and the cholesterol determinations the finely ground tissue was extracted with the alcohol-ether mixture. For this the tissue did not need to be frozen *in situ*. Generally we used 50 cc. of the Bloor mixture to extract a gram of tissue, repeating the extraction three times. The analysis of the lipid-P and the cholesterol was carried out in the same manner as in the case of the blood extracts.

#### MUSCLE GLYCOGEN

Since glycogen plays such an important role in muscle metabolism we turned our attention first of all to this substance. In table 4 the results of the determinations of muscle glycogen are reported. The average glycogen content in normal rabbits (after 1 day of fasting) was found to be 556 mg.% whereas the average for eighteen dystrophic animals was only 119 mg.%.

This enormous loss of glycogen in the dystrophic muscles is not a starvation effect. In a normal rabbit fasting 3, 6 and even 14 days we still found 358, 273 and 149 mg.% glycogen, respectively. Although the diseased animals do partake sparingly of food, none of the dystrophic rabbits naturally undergo anything approaching such a degree of inanition. The data in table 4 have been arranged in the order of a diminishing glycogen content of the muscles and comparing these with the findings of the histological examination of the muscles it is seen that a close relationship exists between the degree of degeneration and the loss of glycogen. However, neither the relative nor the absolute loss in weight, nor the length of time during which weight was lost, indicates the degree of dystrophy or the extent of the change in glycogen content. Further evidence for the correlation between the glycogen content and the extent of the muscular degeneration is found in the study of the two rabbits (213 and 280) which

have recovered from the attack of dystrophy and in which a muscle glycogen content of 308 and 273 mg.%, respectively, was observed.

TABLE 4

*Muscle glycogen in normal and dystrophic rabbits (milligrams per 100 gm. fresh tissue)*

RABBIT	LOSS OF WEIGHT IN GRAMS	PER CENT LOSS OF WEIGHT	DAYS ON DIET	GLYCOGEN (AS GLUCOSE)	CONDITION OF RABBIT
223	0	0		716	Normal (fasted 1 day)
307	70	3		596	Normal (fasted 1 day)
215	60	2		476	Normal (fasted 1 day)
281	100	6		434	Normal (fasted 1 day)
303	170	5		358	Normal (fasted 3 days)
253	240	9		273	Normal (fasted 6 days)
206	750	32		149	Normal (fasted 14 days)
213	100	5		308	Recovered
280	20	1		232	Recovered
292	240	22	33	218	Dystrophic (diet 13 + magnesium gluconate)
254	190	16	32	209	Dystrophic
226	250	30	31	187	Dystrophic
296	80	5	54	150	Dystrophic (+)
208	80	3	113	143	Dystrophic (++)
306	270	13	72	141	Dystrophic
256	400	31	33	139	Dystrophic
279	420	28	54	139	Dystrophic
285	450	29	43	126	Dystrophic (++++)
227	250	28	37	113	Dystrophic
286	230	17	53	109	Dystrophic (++++)
209	45	7	28	107	Dystrophic
258	220	17	30	93	Dystrophic
266	530	29	58	92	Dystrophic
313	390	31	37	83	Dystrophic
288	530	26	66	81	Dystrophic (++++)
263	260	20	47	81	Dystrophic (+++)
260	460	20	40	74	Dystrophic (+++)
275	400	25	53	69	Dystrophic

These studies suggest a possible relationship between the carbohydrate metabolism and the development of the muscular dystrophy. The question has naturally arisen as to whether it would be practicable to prevent or at least to alleviate the disease by maintaining the muscle glycogen depot

intact. Franke ('34) reported that the muscle and liver glycogen content can be materially increased by the administration of magnesium gluconate. We performed a number of experiments feeding rabbits this substance together with the dystrophic diet 13 but this did not affect either the severity or the onset of muscular dystrophy in the test animals. In rabbit 292, belonging to this series, we found a fairly high glycogen content (218 mg.%) at the time when the dystrophy had developed. Obviously, the loss of muscle glycogen is not the cause but rather a symptom of the dystrophic reaction.

#### ACID SOLUBLE PHOSPHORUS COMPOUNDS

The acid soluble phosphorus compounds constituting such an essential link in the chemical mechanism of muscular activity, we anticipated that an investigation of the changes occurring at the onset of dystrophy would yield significant data for an interpretation of the nature of the dystrophic process. To facilitate the survey of these results we present graphically in figure 2 the absolute as well as the relative distribution of the phosphate compounds in normal and in dystrophic rabbit muscles and for purposes of comparison we also give the analytical data gleaned from the literature. The results we found for the normal rabbit muscle are in good general agreement with those reported by other investigators. The results obtained with the muscle from dystrophic animals show considerable individual variation, as might be expected. However, the total acid soluble-P is noticeably reduced in most cases analyzed, and the decrease generally is proportional to the extent of the degeneration determined histologically. But though the absolute quantity of the acid soluble-P and of its various fractions is decreased, the relative distribution of the different P fractions is hardly at all affected. The per cent of phosphagen in both normal and dystrophic muscles is the same, the inorganic phosphate and the undetermined acid soluble-P fraction is somewhat greater and the hexosemonophosphate is smaller in the degenerated muscles. However, even these small changes still fall within

the range of normal variations. In this respect our results on the rabbit muscles are in good agreement with the results obtained by Nevin ('34) on biopsy material from human myopathy cases.

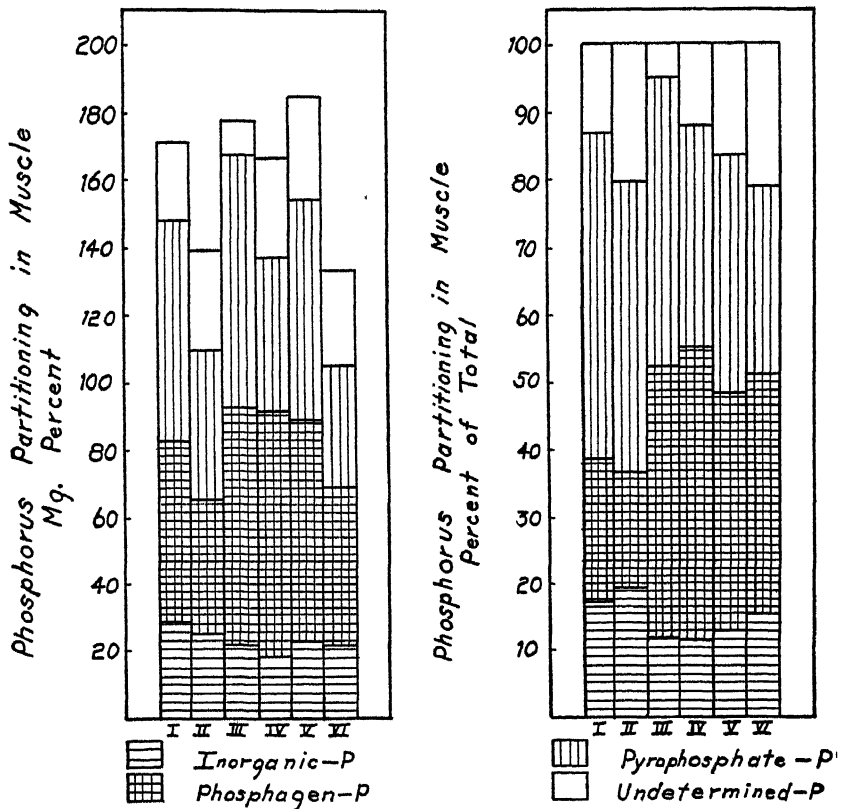


Fig. 2 The absolute and relative partitioning of the acid soluble phosphorus compounds of muscles from normal and dystrophic rabbits. Columns I, II, III and IV are based on the results of analyses of normal rabbit muscles obtained from the literature (Kerr and Blish, '32; Milroy, '31; Nevin, '34; Sacks and Sacks, '33). Columns V and VI are based on our own analyses of normal and dystrophic rabbit muscles, respectively.

The fact that the relative distribution of the various P fractions in muscles from normal and from dystrophic rabbits is essentially similar fits in well with the histological picture of the muscles. Even in muscles affected by extensive degeneration normal fibers are found amidst the debris of de-

generated fibers. The acid soluble-P compounds being intracellular components, the normal relative distribution would indicate that in the intact muscle fibers the chemical structure is likewise maintained intact, but the decrease in the total acid soluble-P content results from the disintegration of the degenerated fibers and is, therefore, proportional to the extent of the dystrophic change.

#### MUSCLE CREATINE

Goettsch and Brown ('32) studied the creatine content of dystrophic muscles and found that it decreased in proportion to the degree of degeneration.

During our investigation only a few determinations of muscle creatine were made and these confirm the findings of Goettsch and Brown. In muscles, which on microscopic examination showed marked degeneration, the directly determined total creatine fell below 100 mg. %.

We also calculated the creatine from the phosphagen-P content of the muscles. In the muscles from normal rabbits about 60% of the total creatine was in the combined form as phosphagen, while in the muscles from dystrophic rabbits this value rose to 76% of the total. Realizing the error inherent in the creatine determinations, we nevertheless think that the difference in these values is too large not to have a significance. From our analyses we conclude that a greater proportion of the muscle creatine is in the esterified form as phosphagen in the dystrophic than in the normal muscle. This may perhaps be interpreted to indicate a compensatory adjustment in the chemical structure of the muscle.

#### CHOLESTEROL

In view of the pronounced hypercholesteremia which we observed in the dystrophic rabbits it seemed advisable to investigate the cholesterol content of the muscles. Later, when we discovered that the cholesterol of the degenerating muscles is two and three times as great as that of the normal muscles, we extended our investigation to include not only the



gastrocnemius muscle but several other skeletal muscles as well as smooth muscles, the heart, and the various visceral organs and brain. The results of this study are summarized in table 5.

All the skeletal muscles examined, the gastrocnemius, gluteus and the muscles of the foreleg from dystrophic rabbits show a greatly increased cholesterol content. The abdominal and the intercostal muscles also show a definite, though not as pronounced, increase in the cholesterol content.

TABLE 5  
*Summary of tissue cholesterol studies*

TISSUE	NORMAL		DYSTROPHIC		DIFFERENCE IN MILLIGRAMS PER CENT	PER CENT CHANGE
	Number of determi- nations	Average milligrams per cent cholesterol	Number of determi- nations	Average milligrams per cent cholesterol		
Gastrocnemius	6	65	7	189	+ 124	+ 191
Gluteus	6	54	4	99	+ 45	+ 83
Foreleg muscle	5	67	4	115	+ 48	+ 72
Heart	4	124	2	124	0	0
Abdominal muscles	2	53	2	78	+ 25	+ 47
Intercostal muscles	2	79	2	104	+ 25	+ 32
Intestines and stomach	4	165	2	170	+ 5	+ 3
Kidney	4	320	2	332	+ 12	+ 4
Lung	5	502	3	432	— 70	— 14
Liver	5	320	4	294	— 26	— 8
Brain	5	1866	4	1914	+ 48	+ 2
Spleen	3	414	3	329	— 85	— 20

The heart, stomach, kidney and brain exhibit practically no variation so far as the cholesterol is concerned from that found in the normal rabbits. This is especially true for the heart. In the case of the liver, lung and spleen the cholesterol content in the dystrophic animals is less than in the normal ones, the relative decrease being smallest in the liver and largest in the spleen.

This very striking and enormous increase in the cholesterol content of the skeletal muscles from dystrophic rabbits, coupled with the observation that the cholesterol content of other muscular structures (heart, smooth muscle) and of

several organs remains practically unchanged, suggests a severe disturbance in the cholesterol metabolism originating primarily in the muscle tissue itself. The limitation of the significant cholesterol changes to skeletal muscle places nutritional dystrophy more definitely among the myopathic disorders.

The fact that the heart from dystrophic rabbits has a normal cholesterol content fits in well with the findings of Goettsch and Pappenheimer ('31) that the cardiac muscle in such animals is also normal from the morphological standpoint.

#### SUMMARY

1. In rabbits suffering from nutritional muscular dystrophy we do not find the characteristic zig-zag sugar tolerance curve generally occurring in human subjects with muscular dystrophy. The glycemic curve of the dystrophic rabbit differs, however, from that of the normal rabbit in that it rises slower, reaching a peak in about twice the length of time, does not attain such a high level and slopes off more gradually than in the latter.

2. The analyses of a number of fasting bloods from both normal and dystrophic rabbits fail to indicate any appreciable variation between the two groups so far as the sugar, lactic acid, total acid soluble phosphorus and the partition of its several fractions are concerned.

3. The lipid-P and cholesterol content of the blood show marked increases in the diseased animals, the increase being proportional to the degree of the dystrophic change. When by proper dietary means the regenerative process in the dystrophic muscle is initiated leading to recovery from the disease the concentration of the lipid-P and particularly of the cholesterol in the blood decreases again, returning ultimately to the normal values.

4. The glycogen content of the skeletal muscles from rabbits suffering from nutritional muscular dystrophy is very greatly reduced, the reduction being proportional to the extent of the degenerative process.

5. The absolute concentration of the acid soluble phosphorus and its different fractions is decreased in the dystrophic muscles, the decrease being proportional to the degree of dystrophy, but the relative distribution of the various phosphorus fractions is practically unaltered and is nearly the same as in the normal muscles.

6. The creatine content of the muscles diminishes parallel to the development of dystrophy but the portion of the total creatine esterified in the form of phosphagen is considerably greater in the dystrophic than in the normal muscles.

7. The cholesterol content of the skeletal muscles is greatly increased in dystrophic rabbits. In the muscles of the extremities the cholesterol content may become even more than doubled while in the muscles of the trunk it is not so large. The cholesterol content of the muscles from visceral organs (heart, stomach, intestine) is practically unchanged, which is true also for the brain and kidneys, but in liver, lung and spleen there is a variable but definite decrease.

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# METABOLISM STUDIES IN NUTRITIONAL MUSCULAR DYSTROPHY

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SIX FIGURES

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The numerous biochemical investigations of the clinical myopathies indicate that there is a fundamental change in the metabolism of the dystrophic organism. The creatine-creatinine metabolism has received the most attention in these studies, although investigations of the most diverse nature may be found in the literature.

It was considered advisable to study the metabolism of our experimental animals (Morgulis and Spencer, '36) in an effort to determine the fundamental changes which accompany nutritional muscular dystrophy. We studied animals on control diets, on the basic dystrophy producing diet 13 (Goettsch and Pappenheimer, '31), and on the basic diet until the manifestations of dystrophy became evident when this was changed to a recovery diet. Daily records were kept of the body weight of each rabbit; of the weight of food and water consumed; and of the volume, reaction and specific gravity of the urine voided. The urines were analyzed for total nitrogen, creatinine, creatine, phosphorus, chloride and organic acids.

## METHODS

The cages used for the metabolic study of rabbits were specially designed, and all the metal parts were chrome-plated. A 10% thymol solution in chloroform was used as a preservative for the urine. The apparatus was cleaned

daily. The urine specimen was acidified with a few drops of  $\text{H}_2\text{SO}_4$  (which prevented the escape of ammonia and dissolved any precipitated phosphates) and made up to a volume of 150 cc. with the cage washings.

The total nitrogen, creatine and creatinine, and chlorides were determined according to the directions given by Folin ('34). The phosphorus was determined by the Fiske and Subbarow ('25) method and organic acids by Greenwald's ('29-30) modification of the familiar Van Slyke-Palmer procedure.

#### EXPERIMENTS ON THE CONTROL DIETS

Three different control diets, namely, rabbit-chow, diet 11 and diet 201 were used. Rabbit-chow was chosen since it has been found to be a well-balanced food for rabbits; diet 201 (rabbit-chow treated with ethereal-ferric chloride) was fed to ascertain the effect of ethereal-ferric chloride treatment, per se; while diet 11 (untreated diet 13) was used because of its close similarity to diet 13, the basic dystrophy producing diet.

The results of the experiments on the control diets are presented in figures 1, 2 and 3 and a summary of the metabolism experiments is presented in table 1. Rabbits 306, 311 and 310 maintained on rabbit-chow, diet 11 and diet 201, respectively, appeared perfectly normal throughout the experimental periods. The most rapid gain in weight was made on diet 11, while the slowest gain was obtained on diet 201. Although the water consumption of the three animals varied, in every case the volume of urine voided was very nearly one-half the total water intake. The urine of all three rabbits was alkaline, except for a 2-week span during which the urine of rabbit 310 was neutral to litmus.

The constancy of the elimination of various urinary constituents on these widely different diets was surprising. The most radical difference was found in the case of phosphorus, since none was excreted in the urine on either rabbit-chow or diet 201 (treated rabbit-chow), while an average daily excretion of 38 mg. per kilogram body weight was found in the

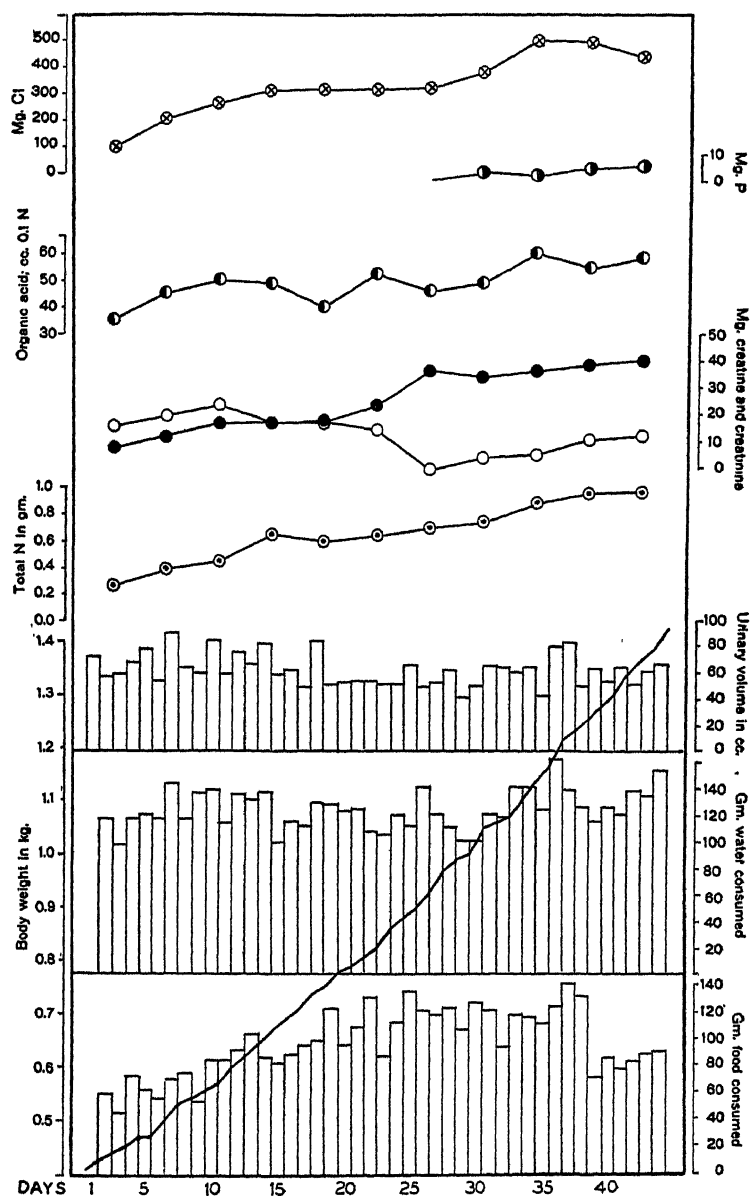


Fig. 1 Metabolism studies on rabbit 306 maintained on rabbit-chow as a control diet.

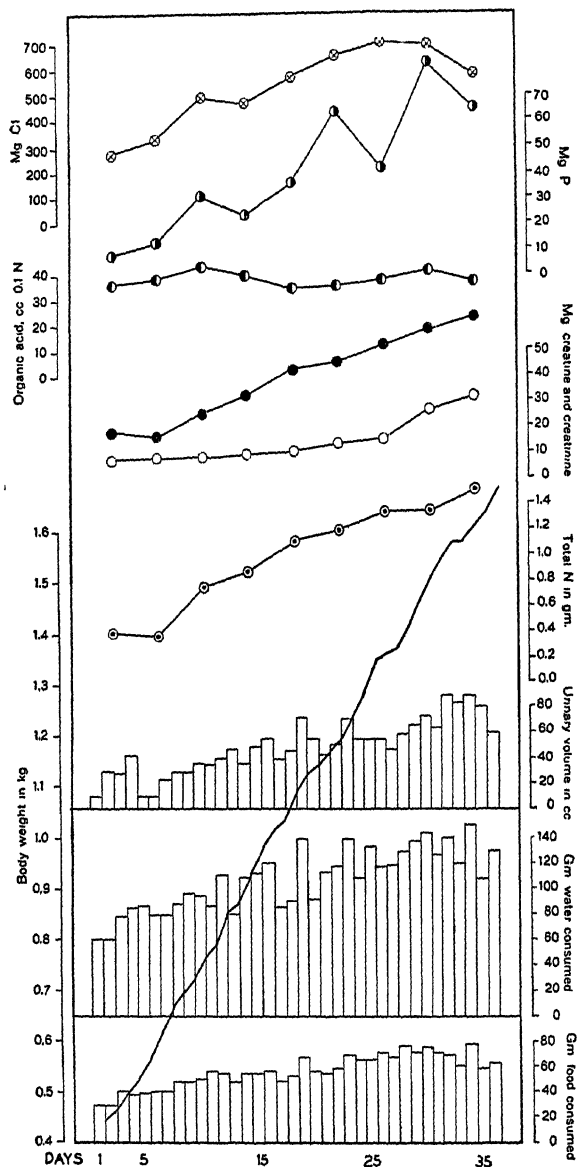


Fig. 2 Metabolism studies on rabbit 311 maintained on diet 11 as a control diet.



animal on diet 11. The chloride excretion was greatest on diet 11 in spite of the fact that diet 201 had been treated with ethereal-ferric chloride, while the lowest chloride excretion was obtained on rabbit-chow.

In considering the nitrogenous constituents, it was found that the greatest positive nitrogen balance was obtained on

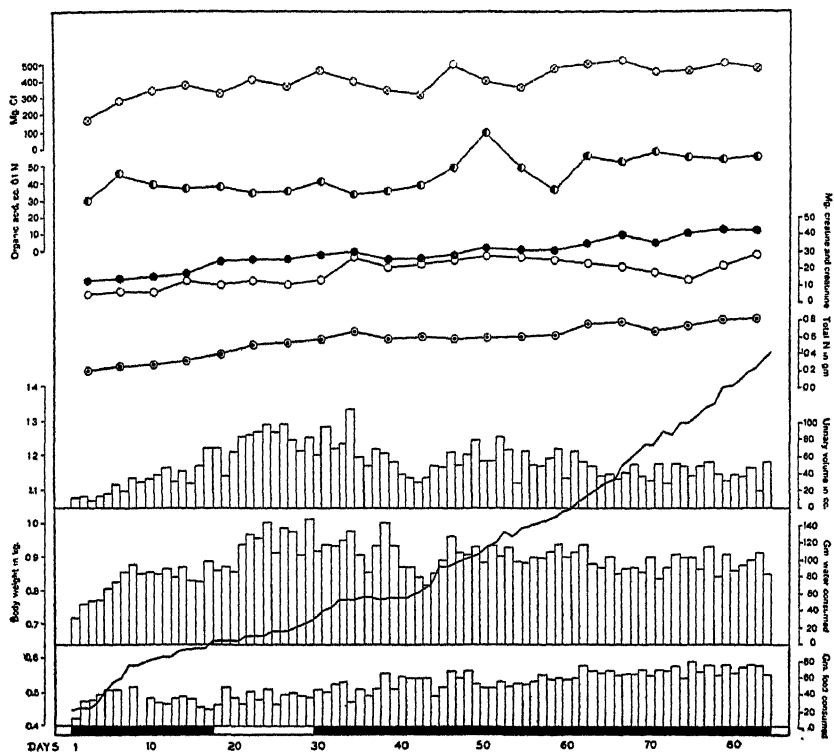


Fig. 3 Metabolism studies on rabbit 310 maintained on diet 201 as a control diet.

rabbit-chow, while the lowest was found on diet 201. This low balance may, possibly, be linked with the exceedingly slow rate of growth given on this diet. Turning to the creatinine, it may be noted that its excretion was quite constant, irrespective of the diet. The average daily excretion of creatinine was 34, 37 and 31 mg. per kilogram body weight on each of the three diets, giving an average for the normal

rabbit of 34 mg. per kilogram. Although the creatine excretion fluctuated more than that of creatinine, nevertheless, its daily excretion in the control rabbits averaged about 17 mg. per kilogram. The creatinine N, calculated as per cent of total N, was also rather constant in the three control rabbits, the highest value being obtained on diet 201 and the lowest on diet 11. Similarly the per cent of creatine N, was greatest on diet 201 and least on diet 11.

In spite of the small daily variations, the urinary excretion, irrespective of the diet, manifested a striking constancy in all our normal control rabbits.

#### EXPERIMENTS ON A DYSTROPHY PRODUCING DIET (DIET 13)

The results obtained with normal rabbits are in sharp contrast to those found in the study of dystrophic animals. Rabbit 274 was the first dystrophic animal studied. This animal made a steady gain of weight on diet 13 until the thirtieth day, when it showed a wavering in its growth curve. At this point the rabbit was placed in a metabolism cage and kept there until death from advanced dystrophy occurred. The data obtained during this period of dystrophic changes are recorded in figure 4.

The wavering of the body weight curve is an indication of approaching dystrophy. On the thirty-seventh day there was a sharp break in weight, which we designate as the 'critical point.' From this moment on the animal lost weight very rapidly, the average loss being 45 gm. per day, and the total loss was almost 25% of the maximum weight. The earliest outward signs of dystrophy became apparent in this rabbit 3 days after the sharp break in body weight (thirty-ninth day on diet 13) and from thence on developed rapidly until at the time of death the manifestations of dystrophy were manifold.

The sharp break in the weight curve is a very characteristic event in the development of dystrophy. We designate this as the 'critical point' because from this time on the dystrophic process runs a very acute course, soon terminating in death. Although in the matter of details no two rabbits

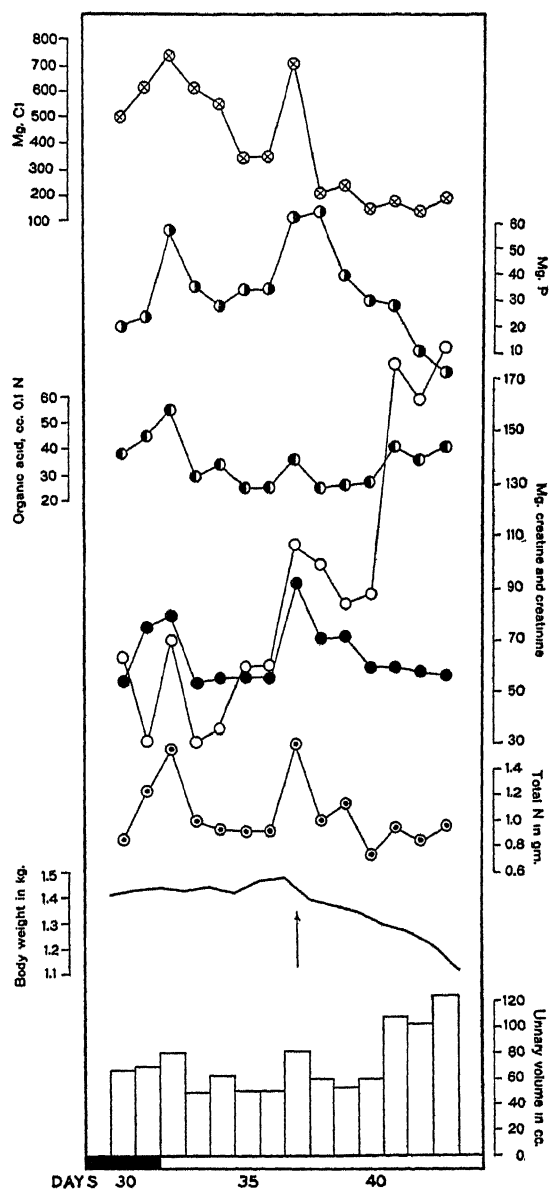


Fig. 4 Metabolism studies on rabbit 274 maintained on the dystrophy producing diet 13.

are alike, all rabbits show the same general behavior, of which the 'critical point' is a very significant landmark. Recovery from dystrophy becomes more difficult and finally impossible the longer the animal is carried beyond the 'critical point.'

The urine was definitely alkaline until the wavering in the body weight curve occurred, at which time it became definitely acid and remained so until death. This shift to an acid reaction took place 5 days before the 'critical point,' 8 days before the dystrophy became outwardly apparent, and 12 days before death. Throughout this period the volume of the urine remained practically constant except for a slight diuresis at the 'critical point' and a more marked diuresis the last 3 days before death. At the time of the 'critical point' there was, however, a sharp rise also in the excretion of total nitrogen, creatinine and creatine. At that time the excretion of creatine exceeded that of creatinine, and this excessive creatinuria became more pronounced as the symptoms of dystrophy developed, so that the last 3 days before death the urinary creatine output was four to five times as great as that prior to the onset of dystrophy. At the 'critical point' the phosphate and chloride excretion likewise increased but, after this initial increase, the excretion of both of these urinary constituents decreased as the dystrophy progressed. Throughout the entire experimental period the organic acid excretion was practically constant.

The results obtained with rabbit 274 during the development of dystrophy may be compared with those obtained with the normal control rabbits (table 1). The average daily loss in weight was 22 gm., or almost equal to the average gain in weight of a rapidly growing, young rabbit. Unfortunately no record of the food and water intake of this animal was kept, and we cannot, therefore, determine the relationship of the urine volume to the water intake nor to the nitrogen intake.

Regarding diet 11 as the control diet for diet 13, the excretion of phosphorus and chloride was diminished in the dystrophic animal. The creatinine excretion was increased by about 30%, while the creatine output increased enormously, at least

200 to 300%. Likewise, the creatinine N, calculated as per cent of total N, increased a little but the creatine N increased two- to threefold. Thus, in the definitely dystrophic animal the striking change is the enormous increase in creatine excretion, accompanied by a lowered excretion of phosphorus and chloride.

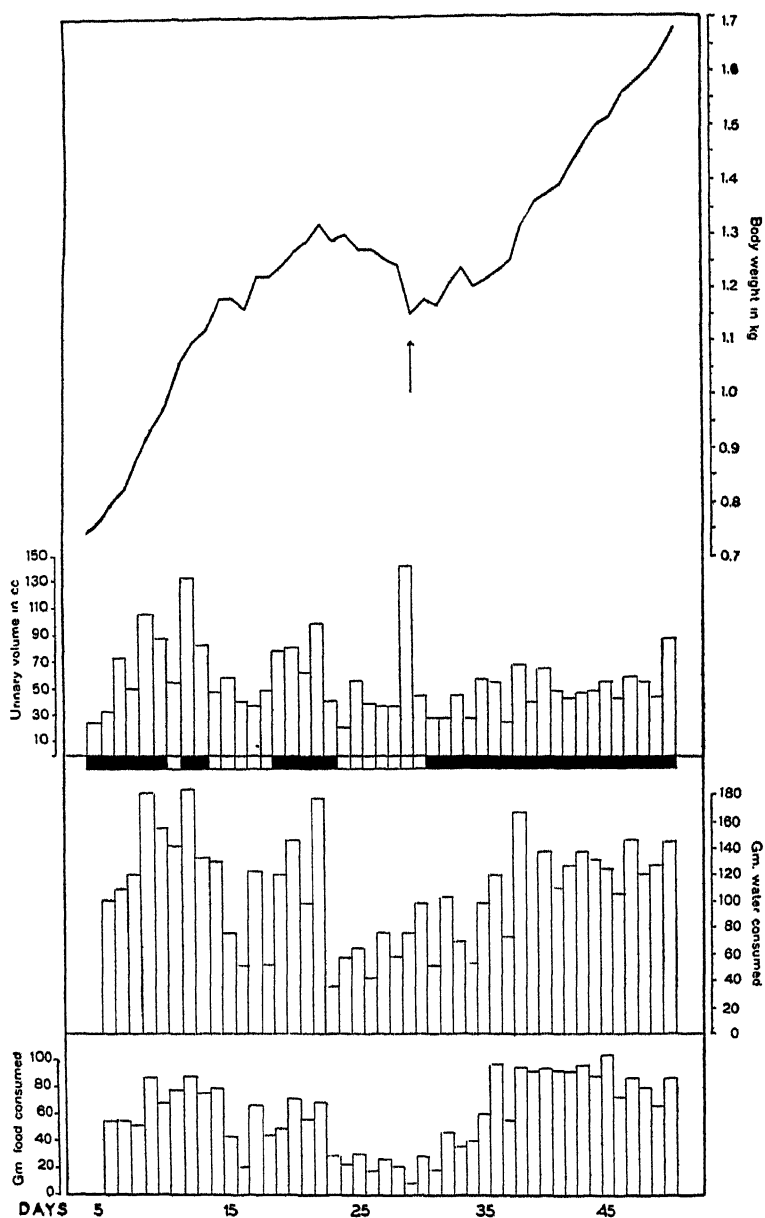
#### EXPERIMENTS ON DIET 13 FOLLOWED BY RABBIT-CHOW

The most interesting results were obtained in the metabolic study of rabbit 303, recorded in figures 5 and 6. This animal was maintained on diet 13 until it showed pronounced signs of dystrophy and had definitely reached the 'critical point,' when it was changed over to rabbit-chow. It was kept on this diet until complete recovery from the dystrophy was attained.

The data obtained in the experiment with rabbit 303 are summarized in table 1. The experimental period on diet 13 is divided into two parts, IA and IB. Period IA covers the period of active growth on diet 13, while period IB includes the period of wavering and decreasing weight. In order to appraise the metabolic changes after definite signs of dystrophy had developed, the results of the last 3 days on diet 13 are grouped separately as period IC. Likewise, the recovery period is divided into IIA, the period of readjustment and slow gain, and IIB, the period of rapid gain during which the animal appeared practically normal.

During the IA period the animal consumed the largest amount of water, but as dystrophy developed (period IB), the water consumption gradually diminished. At first the volume of urine excreted was practically half of the water consumption but during IB the decrease in urine volume did not keep pace with the diminished water consumption, so that finally the urine volume equalled the volume of water taken in. Upon recovery, the usual ratio of 1:2 between the urinary output and water intake was rapidly restored.

As the animal became dystrophic its food consumption gradually decreased. However, its nitrogen excretion did not



Figs. 5 and 6 Metabolism studies on rabbit 303 in which dystrophy was produced by means of diet 13 and then recovery effected by the feeding of rabbit-chow.

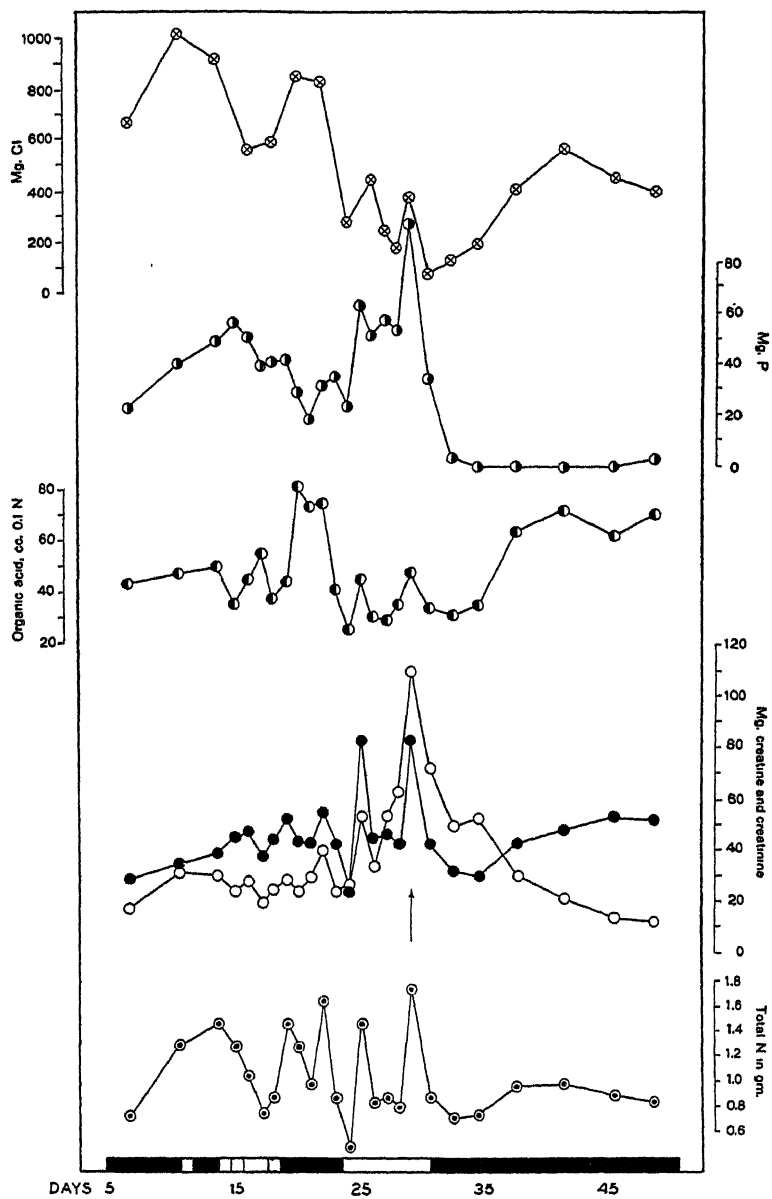


Figure 6

decrease to the same extent, so that during the period IC, a definitely negative nitrogen balance was established. Again, upon recovery, the nitrogen balance gradually approached the same value found in the control rabbit.

As the rabbit passed through periods IA and IB its creatinine excretion increased only slightly and even during IC no marked rise was noted. During the recovery period the creatinine excretion rapidly approached that in the control rabbit. The average daily excretion of creatinine during this period was 30 mg. per kilogram as compared with 34 mg. for the control animal. In contrast to creatinine, the creatine excretion increased enormously as dystrophy developed. However, the creatine output also decreased and quickly approached the normal value upon recovery of the animal. The average daily excretion of creatine in the recovery period was 19 mg. as compared with 17 mg. per kilogram for the normal rabbit. The per cent of creatine N (on the basis of the total N excretion) showed a great increase, while that of the creatinine N showed only a relatively small increase, but these values returned to the normal level during the recovery period.

#### SUMMARY OF METABOLISM EXPERIMENTS

The metabolism experiments have shown that pronounced changes take place in the metabolism of the dystrophic organism. For the sake of clarity, the results will be summarized by citing the changes which take place during definite stages in the course of the disease.

1. During the period of approaching dystrophy: The weight curve wavers or drops slowly, while the consumption of food and water becomes less. The urine excretion also decreases and it becomes acid in reaction. The urinary chlorides decrease gradually while the phosphorus elimination increases. The total nitrogen excretion decreases somewhat, but the creatinine excretion remains practically constant while that of creatine increases by leaps and bounds.

2. At the 'critical point': The body weight declines sharply. The ratio of urine output to the water intake approaches the



TABLE 1  
Summary of metabolism experiments

RABBIT	DIE	DAYS ON DIET	REMARKS	AVERAGE WEIGHT IN GRAMS	AVERAGE DAILY GAIN OR LOSS IN WEIGHT IN GRAMS	FOOD CONSUMPTION GRAMS/DAY/KILO- GRAMS	WATER CONSUMPTION GRAMS/DAY/KILO- GRAMS	URINE OUTPUT CENTIMETERS/DAY/ KILOGRAMS	DAILY URINARY EXCRETION							
									Total N grams/ kilograms	Oreatinine milligrams/ kilograms	Oreatinine milligrams/ kilograms	Total P milligrams/ kilograms	Total Cl milligrams/ kilograms	N Balance grams/ kilograms	Oreatinine-N per cent of total N	Oreatinine-N per cent of total N
306	Rabbit- chow	44	Normal	850	+ 22.0	113	141	70	0.762	34	19	0	382	+ 1.7	1.5	0.8
311	11	36	Normal	1080	+ 34.4	52	102	46	0.930	37	13	38	537	+ 1.1	1.3	0.5
310	201	84	Normal	900	+ 12.5	55	110	55	0.620	31	20	0	456	+ 0.6	1.7	1.1
Average				943	+ 23.0	73	118	57	0.771	34	17		458	+ 1.1	1.5	0.8
274	13	14	Period of definite dystrophy	1300	— 22.0			56	0.800	49	68	25	302		2.0	2.9
303	13	25	Period I dystrophy	1130	+ 15.6	43	89	55	0.970	37	29	36	571	+ 0.7	1.3	1.0
303	13	10	Period IA	964	+ 34.0	72	141	72	1.030	34	27	36	908	+ 1.8	1.1	0.9
303	13	15	Period IB	1245	— 2.0	31	67	47	0.865	39	30	37	410	+ 0.4	1.5	1.2
303	13	3	Period IC (last 3 days of period IB)	1213	— 33.0	14	56	56	0.895	45	107	55	220	— 0.3	1.9	3.3
303	Rabbit- chow	21	Period II recovery	1450	+ 25.2	50	70	35	0.610	30	19	1	265	+ 0.5	1.7	1.0
303	Rabbit- chow	8	Period IIA	1210	+ 12.5	40	72	36	0.680	30	36	5	203	+ 0.2	1.5	1.8
303	Rabbit- chow	13	Period IIB	1575	+ 33.0	58	87	35	0.600	32	11	Trace	309	+ 0.7	1.7	0.6

value of 1:1 as the acidotic metabolism develops. There is marked diuresis accompanied by a relatively slight increase in creatinine, total nitrogen, phosphorus and chloride excretion and an enormous increase in the creatine elimination. At this point the creatine N, calculated as per cent of total N, doubles or trebles itself and the nitrogen balance becomes definitely negative.

3. During the period of progressive dystrophy, i.e., from the 'critical point' to the time of death: The weight continues to fall rapidly. The phosphorus and chloride excretion gradually decrease. The creatinine and total nitrogen decrease from the high level reached at the 'critical point,' while the creatine excretion continues to rise till the time of death. The urine volume decreases following the brief strong diuresis at the critical point, but just before death a marked diuresis occurs again.

4. During the period of recovery, following a change of diet at the 'critical point': The weight increases at first slowly, then very rapidly. The food and water intake increases gradually. The ratio of the urine output to water intake soon returns to the normal value of 1:2. The urine almost immediately becomes alkaline. The excretion of phosphorus and chloride approaches the values normal for that diet. The nitrogen balance becomes positive and the creatine elimination approaches the normal level. Meanwhile, the rabbit gradually loses the symptoms of dystrophy and behaves perfectly normal.

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# THE EFFECT OF SELENIUM CONTAINING FOOD-STUFFS ON GROWTH AND REPRODUCTION OF RATS AT VARIOUS AGES<sup>1</sup>

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## THREE FIGURES

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In the assay of grain samples (Franke, '34) it was observed, that rats which were several days older than the other rats in the group usually survived much longer on the toxic diet. In this report it was stated that 71.1% of the rats placed on lethal samples of grain were dead by the sixtieth day of the experiment. The rats were placed on experiment at ages varying from 27 to 31 days.

In a preliminary trial (series 69) to test out the theory that older rats were much more resistant to the toxicant, three adult male rats were placed in a cage and given the standard diet made with no. 582 wheat (lethal). The animals were sacrificed on the 60th, 120th and 360th days of the experiment. No outstanding gross pathology was present in these rats.

Another group (series 76) of six males was selected and placed on a control wheat diet. They were then shifted to the no. 582 wheat diet at the ages of 60, 70, 80, 90 and 100

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days, respectively. All of the rats showed appreciable weight losses for the first few days after the change in diet. Growth was then resumed, but at a somewhat depressed rate. The animals were sacrificed after 360 days on the diet. None of the rats had died. Post mortems revealed varying degrees of atrophy and regeneration in the liver. None of the rats were anemic.

A group of very young rats was placed on the toxic diet (no. 582 wheat) at the age of 20 to 22 days (series 90). Death occurred with acute symptoms in four out of five cases before the twenty-second day of experiment. The remaining rat died on the fifty-second day with distinct atrophy of the liver.

These preliminary trials showed that a marked increase in resistance occurred between the twentieth and the sixtieth day of life in the case of the rat. A long-time experiment (series 99) was accordingly planned to show the effects of the toxicant on rats from weaning age to maturity.

#### EXPERIMENTAL

Forty-two rats were weaned when 21 days of age and placed in seven groups of six rats each (two males and four females). The rats were also divided as evenly as possible according to litter and weight, and placed in individual drawer cages.

Group I was shifted at once to the toxic no. 582 wheat diet (24.6 ppm. of naturally occurring Se). The remaining six groups were placed on the control wheat diet. Groups II, III, IV, V and VI were shifted to the toxic diet when the rats were 42, 63, 84, 105 and 186 days of age, respectively. Group VII remained on the control diet throughout the experiment. All of the rats served as controls until they were shifted to the toxic diet.

After each group had been on the toxic diet for a period of 40 days, the rats were placed in large breeding cages for a period of 14 days after which they were replaced in their individual cages.

During the breeding period the males on the toxic diet were placed with stock females from 1 to 8 p.m. daily, the stock diet being removed. It was thus possible to mate the males with both affected and normal females. Group VI was used as a control for the matings during the period from 82 to 131 days of age, while group VII was used during the period of 145 to 226 days of age. By this time group VI had served its purpose in controlling the earlier experimental matings and was now shifted to the toxic diet, and 40 days later the rats were mated, using group VII for controls.

In order to make a cross breeding of control males with affected females another series of four groups of four females (series 117) was fed and handled to correspond to groups II, III, IV and V of series 99.

The rats in both series were weighed at 5-day intervals, and food intake was recorded daily. Fresh distilled water containing a trace of iodine was available at all times. The composition of the diet has been given in a former publication (Franke and Potter, '34). Careful autopsies were made of all rats at death.

#### DISCUSSION OF RESULTS

The growth of the animals is shown in figures 1 and 2. The rats in group I which were placed on the toxic diet at the age of 21 days were completely unable to withstand the toxic effects of the diet. All were dead by the twenty-first day of experiment. The average survival was 13 days. All of the animals showed acute symptoms (Franke, '34).

In striking contrast to the animals in group I, the animals in group II, which were placed on the toxic diet at the age of 42 days, not only failed to die in the time which caused death for group I, but were still alive after 40 days. Franke ('34) showed that in the case of 325 rats which were placed on lethal grain samples at the age of 28 days, 38.5% were dead by the fortieth day. Thus it is apparent that a marked increase in resistance to the toxicant occurred between the twenty-first and forty-second day of life, and much of this

between the twenty-eighth and forty-second day. Group II was nevertheless greatly affected by the toxicant and responded by an immediate restriction of food intake (fig. 3) and a sharp loss in weight (figs. 1 and 2). After the preliminary weight loss an equilibrium was apparently reached,

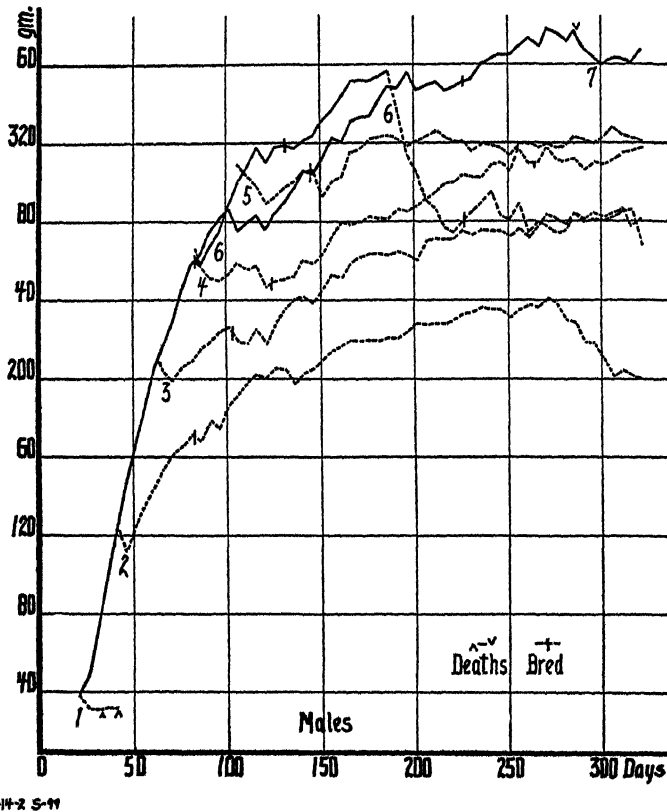
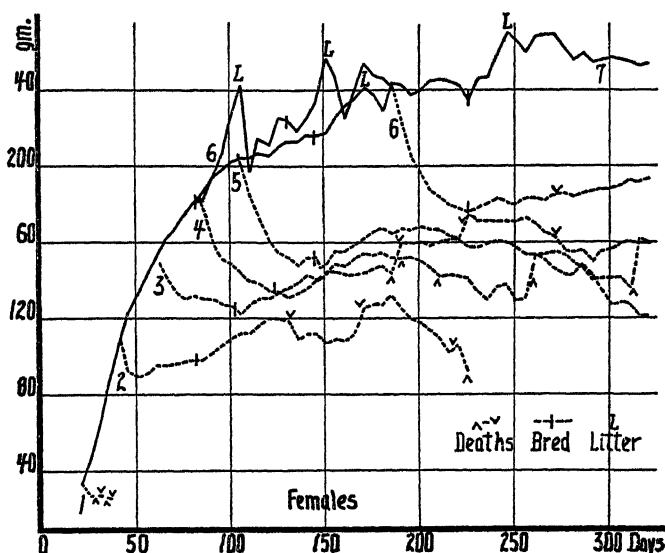


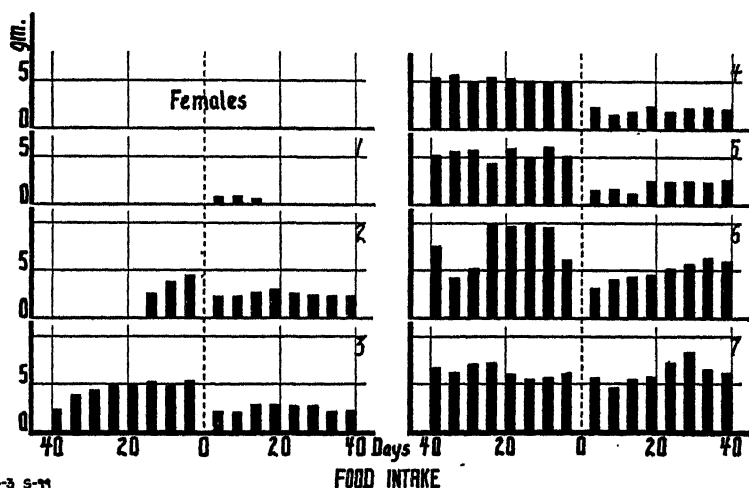
Fig. 1 Growth of male rats. Showing effect of toxic diet on rats of various ages, as follows: Group 1, 21 days; 2, 42 days; 3, 63 days; 4, 84 days; 5, 105 days; and 6, 186 days old. Average weight of two.

and the rats continued at a subnormal and irregular rate of growth, with all dead by the 225th day. The behavior of the remaining groups was strictly analogous to that of group II, except for longevity of life.



R-14-1 5-44

Fig. 2 Growth of female rats. Showing effect of toxic diet on rats of various ages, as follows: Group 1, 21 days; 2, 42 days; 3, 63 days; 4, 84 days; 5, 105 days; and 6, 186 days old. Average weight of four.



R-14-3 5-44

Fig. 3 Average food intake for 40 days before and after shifting from control to toxic diets, with the following exceptions: Group 1 was not on the control diet at all, group 2 was on the control diet less than 40 days, and group 7 was on the control diet throughout.

The results of the various matings are shown in table 1, and show distinctly that the animals on the toxic diets were unable to reproduce. In all matings, in which both males and females had been on the toxic diet, no litters were obtained, while parallel control matings were successful. Particularly

TABLE 1  
*Effect of toxic wheat on reproduction*

GROUP NO.	SERIES 99 AND 117		SERIES 99		SERIES 117, FEMALES MATED WITH STOCK MALES
	Age when shifted to toxic diet (days)	Age when bred (days)	Matings within group	Males mated with stock females	
1	21	Dead at 42			
2	42	82	----- <sup>1</sup>	— <sup>2</sup>	-----
		131	-----	—	
3	63	103	-----	+	+-----
		250			-----
4	84	124	-----	+	++++
		320			+-----
5	105	145	-----	+	+----- <sup>3</sup>
		250			+-----
6	Control	82	++++	Control	
	Control	131	++++	Control	
	186	226	-----	+	
7	Control	145	++++	Control	
	Control	226	++++	Control	

<sup>1</sup> Results are given for each of the four females in the group; + indicates a litter.

<sup>2</sup> Indicates the number of stock females that caste a litter.

<sup>3</sup> One young from this litter reached an age of 61 days, in all other cases pups were either eaten by female or died before twenty-first day.

interesting are the results obtained with group VI. After two uniformly successful matings on the control diet, the group was shifted to the toxic diet. Following the usual 40-day period, the animals were mated again, but the matings were uniformly unsuccessful, no litters being cast. In addition to



the matings between males and females on the toxic diet, the males were mated with normal stock females of proven fertility. The males which were placed on the toxic diet at the age of 42 days were unable to fertilize even the normal females. All of those males which were not placed on the toxic diet until 63 days or more of age were able to fertilize the normal females, although matings with females on toxic diets were unsuccessful. These findings cannot be taken to mean that the females are more susceptible to the toxicants than the males since the affected females were not given the opportunity to mate with normal males.

In the later experiment (series 117) in which affected females were bred by normal males, the matings proved fertile in a few cases. The pups which were born all died or were eaten by the mother soon after birth, except in the case of one litter in the fourth group where one pup lived to 61 days. The growth of the rats in series 117 was almost exactly parallel with that of the animals in series 99.

These results show that animals which receive toxic diets are unable to reproduce successfully. Matings in which both animals have been given toxic diets were completely infertile. Matings in which one of the animals was normal were sometimes fertile, but affected females were unable to raise their young. These results are therefore in agreement with our previous suggestions (Franke and Potter, '35) and the observations of Macomber ('23) who observed that animals of low fertility may yield fertile matings were placed with animals of high fertility, and yield infertile matings with other animals of low fertility.

The older the rats were at the time they were placed on the toxic diets, the longer they survived. The following figures show the marked difference between the survival of the first group and the survival of the remaining groups which were placed on the toxic diet. The average age of the females in the various groups in series 99 are given: I, 32 days; II, 211 days; III, 279 days; IV, 247 days; V, 383 days; VI, 512 days; and VII, 601 days. These averages include the

following survivors which were killed on the 646th day of experimentation: group III, 1; IV, 2; VI, 1 and VII, 4.

Steyn ('34) has discussed the relation between the action of poisons and the age of animals and states that, with the exception of animals in extreme age, the younger the animal the more susceptible it is to the action of poisons. However, MacKay and MacKay ('30) believe that food intake may be a determining factor. Contrary to the results of Cox, Smythe and Fishback ('29) they came to the conclusion that cystine added to the diet is no more toxic for young than for older rats. They considered the apparent difference in effect to be due to the fact that the younger rats ingested more food, and hence more cystine, in relation to their body weight than did older rats. In our own experiments the remarks of MacKay and MacKay would not apply, since the restriction of food intake, which always occurs when toxic wheat is fed, was particularly pronounced in the case of the youngest animals which were placed on the diet. Although inanition undoubtedly hastened the death of the animals in this group, there can be no question but that the younger rats are more susceptible to the toxicant than the older rats.

In checking over the post mortem records from series 99 it was found that very little lung infection occurred before the 300th day of the experiment. Of eighteen rats which died in this period, only two showed definite lung infection, and one of these was a control animal. There was some pleural edema in seven cases. Of the twenty-four rats which died after the 300th day, seven rats, including one control, showed lung infection. Although this data is far from conclusive, it suggests the possibility that the toxicant may render the animals less resistant to infection. The fact that the affected animals are in an extremely poor nutritive condition due to the effects of the toxicant undoubtedly lowers their resistance.

At death the rats in group I showed the typical acute symptoms which have been previously described (Franke, '34). The remainder of the affected rats died with the usual

chronic symptoms. The livers showed atrophy of the central, caudate and left lateral lobes, while the right lateral was hypertrophied in many cases.

The failure of the affected rats to reproduce made it seem quite likely that marked atrophy of the reproductive organs might be found. It is very difficult to make comparisons between the organ weights of normal and affected rats. Even when the weights of the organs are recalculated to 100 gm. of body weight the fact that the controls have body fat while the affected rats are emaciated causes a disparity in the figures. Cases of atrophy are quite likely to be valid, however, since the above effect would be to diminish rather than to increase the differences between the weights of normal and affected organs. A study of the records for the males failed to reveal any marked deviations from the normal. In the case of the females, however, the reproductive organs were definitely under-developed in a number of cases. It appeared that very little development of the female reproductive organs occurred after the females had been placed on the toxic diet. The mating records indicated reproductive failure in cases which were not revealed by the post mortem examinations.

The results of this paper would seem to be of considerable economic importance. Animals which are approaching maturity may have their reproductive powers injured by the feeding of toxic grains, while their weight losses may not be large enough to arouse attention. Furthermore, older animals thrive better on a toxic foodstuff and may show weight gains which cannot be described as subnormal when a control is not present for comparison. Yet the subnormal gains may destroy the margin of profit for the livestock feeder.

## SUMMARY

1. White rats were placed on toxic wheat diets at ages varying from 21 to 186 days, and studied from the standpoint of growth, reproduction, and mortality.

2. There is a rapid increase in resistance to the toxicant between the ages of 21 and 42 days. Animals placed on toxic diets at 21 days lost weight and died in less than 20 days, while rats which were placed on toxic diets at 42 days of age or older lived for more than 200 days in most cases.

3. Rats which were able to survive on the toxic diets for relatively long periods of time showed subnormal growth and a distinct loss in reproductive powers. Matings in which both animals were fed toxic wheat were completely infertile. Matings in which one animal was normal were sometimes fertile, but affected females were unable to raise their young.

4. The economic significance of the results was discussed.

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# THE EFFECT OF THE ADMINISTRATION OF SODIUM BICARBONATE AND OF AMMONIUM CHLORIDE ON THE AMOUNT OF ASCORBIC ACID FOUND IN THE URINE

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TWO FIGURES

(Received for publication May 16, 1936)

In a previous study of the excretion of vitamin C in the urine of normal subjects, individual variations were observed in the response to repeated test doses of ascorbic acid, both during and after apparent saturation with the vitamin (Hawley, Stephens and Anderson, '36). It was suggested that factors not satisfactorily controlled might be responsible for the observed variations. A chance observation in a patient with hemorrhagic nephritis led to the suspicion that the variations in the hydrogen ion concentration of the urine might be accompanied by variations in the urinary excretion of vitamin C. The experiment recorded in this communication was designed to investigate the validity of this hypothesis.

## METHOD

Two normal young men were chosen as subjects. Throughout the duration of the experiments they remained in good health and continued their usual activities as medical students. Except for a short period at the conclusion of the experiment,

<sup>1</sup>Aided by a grant from the California Fruit Growers Exchange

A preliminary report of this investigation appeared in *Proc. Exp. Biol. and Med.*, 1936, vol. 34, p. 218.

a diet practically devoid of vitamin C was taken throughout the period of observation. The daily diet consisted of the following: 50 gm. of crackers, 400 gm. of bread, 27 gm. of cereal, 100 gm. of apple butter, 50 gm. of milk chocolate, 75 gm. of cheese, 50 gm. of butter, 25 gm. of sugar, 1 liter of milk, 126 cc. of 4-X cream, 200 cc. of consomme soup, 90 gm. of beef-steak. To this basal diet measured quantities of vitamin C were added as desired in the form of orange juice, ascorbic acid by mouth or ascorbic acid intravenously,<sup>2</sup> given immediately after breakfast.

Variations in the hydrogen ion concentration of the urine were induced by the ingestion of measured amounts of sodium bicarbonate or ammonium chloride. Twenty-five grams of sodium bicarbonate and 4 gm. of ammonium chloride, taken in divided doses during the day, resulted in the excretion of a markedly alkaline and a markedly acid urine, respectively. No significant changes in the carbon dioxide combining power of the blood resulted from the administration of sodium bicarbonate or ammonium chloride in these amounts. When a vitamin C preparation was given by mouth a period of at least 2 hours was allowed to elapse between the administration of the acid or alkaline salt and the vitamin C preparation.

The urine was collected in 24-hour periods, from 8 A.M. to 8 A.M. Each specimen as voided was immediately divided into two exactly equal portions. One portion, for the estimation of vitamin C, was deposited in a dark brown bottle containing 15 cc. of glacial acetic acid; the bottle was then stoppered and stored in the ice box. We have shown that when urine is stored under such conditions there is less than 5% loss of vitamin C in 24 hours. The second portion of each voided sample was stored in the ice box in a second collection bottle without preservative. At the end of the 24-hour period the hydrogen ion concentration of the pooled sample stored without preservative was determined by the colorimetric method. A number of observations showed that there was no

<sup>2</sup> We are indebted to Merck and Company for providing us with the 'Cebione' (ascorbic acid) for this experiment.

significant change in the pH of the urine when kept for a period of 24 hours under such conditions. The vitamin C content of the acidified sample was determined by rapid titration of the urine against a measured volume of a standardized solution of 2:6 dichlorophenolindophenol according to the method described by Birch, Harris and Ray ('33) and expressed as milligrams per 24 hours.

#### RESULTS

The variations in the urinary excretion of ascorbic acid and in the hydrogen ion concentration of the urine resulting from variations in the intake of vitamin C, sodium bicarbonate and ammonium chloride are shown in figures 1 and 2.

At the beginning of the experiment the 24-hour excretion of ascorbic acid was determined during a single control day on the basal diet. This was followed by the daily administration of 400 mg. of ascorbic acid in the form of 800 cc. of orange juice for a period of 7 days. At the end of this time each subject was excreting approximately 75% of the daily test dose in the urine, indicating that saturation with the vitamin had occurred. Reduction in the daily dose of ascorbic acid to 200 mg. resulted in a comparable reduction in the amount of the vitamin appearing in the urine. The addition of sodium bicarbonate was followed by an increase in the pH of the urine from the control level of approximately 6.5 to approximately 7.0; this was accompanied by a decrease in the urinary excretion of the vitamin to approximately 50% of the test dose. The period between November 30th and December 10th, during which the daily ration of vitamin C was provided as ascorbic acid, is particularly significant. During the period of sodium bicarbonate administration the urine was highly alkaline and contained approximately 50% of the daily test dose. The substitution of ammonium chloride for sodium bicarbonate resulted in the excretion of a highly acid urine and a marked increase in the daily excretion to approximately 100% of the daily dose of vitamin C.

The remainder of the experiment was devoted to observations of the effect of repeated withdrawal and readministration of vitamin C during periods of alkali and acid administration. Under comparable conditions of vitamin intake, the

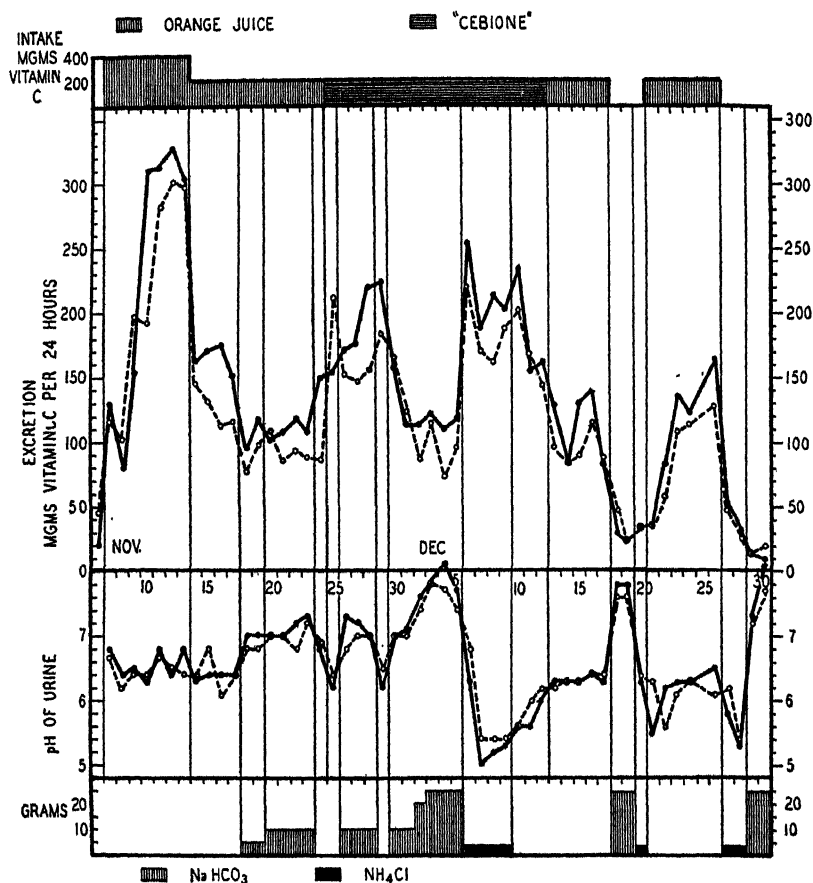


Fig. 1 The effect of the administration of orange juice, crystalline ascorbic acid, sodium bicarbonate and ammonium chloride on the amount of ascorbic acid found in the urine and on the hydrogen ion concentration of the urine. The solid line represents observations in the case of subject L.B. The broken line represents observations made in the case of subject J.F.

urinary excretion of ascorbic acid was consistently greater during periods of ammonium chloride ingestion with a highly acid urine as compare with those periods when sodium bicarbonate was being taken and the urine was highly alkaline.



This held true during periods of depletion as well as during periods of saturation with the vitamin.

At the conclusion of the experiment, both subjects took an uncontrolled diet containing adequate amounts of antiscorbutic foods for a period of 12 days; at the end of this time the excretion of approximately 60 and 100%, respectively, of a test dose of ascorbic acid indicated that a state of saturation

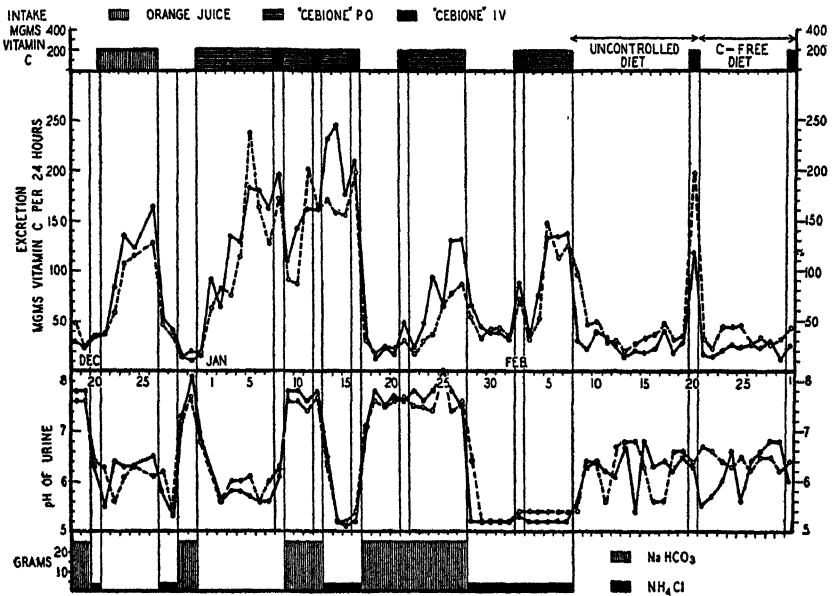


Fig. 2 Legend as in figure 1.

had been maintained by such a diet. During a subsequent 9-day period of vitamin C-free diet the daily 24-hour excretion remained approximately the same as when relatively small but adequate amounts of antiscorbutic foods were being taken. At the end of this period, however, no increase in the control level was observed after a test dose of 200 mg. of ascorbic acid, suggesting that tissue reserves had been depleted.

## DISCUSSION

It may be concluded from the above observations that the urinary excretion of ascorbic acid is increased by the administration of ammonium chloride in amounts sufficient to render the urine highly acid and is decreased by the administration of sodium bicarbonate in amounts which result in a highly alkaline urine. The effect was observed during periods of relative vitamin C depletion and saturation and following the administration of large test doses of ascorbic acid in the form of orange juice, crystalline ascorbic acid by mouth and crystalline ascorbic acid intravenously. It is of interest that Ahmad ('36) has observed an increase in the urinary content of ascorbic acid during the administration of diets high in meat, which might be expected to increase the hydrogen ion concentration of the urine.

At the present time we are unable to offer a satisfactory explanation to account for these variations. Destruction of ascorbic acid in the higher pH ranges during the passage of the urine through the urinary tract would best explain the differences in excretion observed during comparable periods of vitamin C depletion and resaturation. However, it is also possible that the administration of an alkaline salt, such as sodium bicarbonate, may, in some way, result in more efficient storage, greater utilization or destruction, or decreased renal excretion of the ascorbic acid.

Euler, Myrbach and Larsson ('33) have shown that the rate of oxidation of ascorbic acid is greatly increased in the higher pH ranges. In view of the fact that the amounts of sodium bicarbonate and ammonium chloride administered were not sufficient to induce significant changes in the carbon dioxide combining power of the blood, it is considerably unlikely that the variations observed were due to any differences in destruction of the vitamin within the body. The changes in the hydrogen ion concentration of the urine, however, were prompt and striking and closely correlated with the variations in ascorbic acid excretion. It is hoped that a series of animal and in vitro experiments now in progress may help to clarify the

mechanism responsible for the observed effect of the administration of acid and alkali on the amount of ascorbic acid found in the urine.

The practical implications of these observations are limited by the fact that the pH of normal urine, as voided, varies between 4.8 and 7.4, with an average of about 6. The rate of oxidation of ascorbic acid at such hydrogen ion concentrations is relatively slow and the differences in the observed ascorbic acid content of the urine as voided might be expected to be negligible. Under conditions which result in the excretion of a highly alkaline urine, however, the diminution in the amount of ascorbic acid found, determined by the method used in these experiments, may amount to 50%, as compared with that excreted under comparable conditions in a highly acid urine. These findings demonstrate clearly that in evaluating the results obtained by studying the vitamin C content of the urine in any individual, one should know not only the dietary history of that individual but the approximate pH of the urine at the time of study. Discrepancies in findings may be dependent upon changes in the acid base balance of the diet or upon metabolic disturbances which may have a similar urinary effect.

#### SUMMARY

1. The effect of the administration of sodium bicarbonate and ammonium chloride, in amounts sufficient to induce marked changes in the hydrogen ion concentration of the urine, on the excretion of ascorbic acid has been studied in two normal young adults under controlled conditions of vitamin C depletion and saturation.

2. The ascorbic acid content of the urine was consistently and significantly lower during periods of sodium bicarbonate administration, when the urine was highly alkaline, as compared with similar periods of ammonium chloride administration with a highly acid urine.

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# THE RELATION OF SEASON, SEX AND WEIGHT TO THE BASAL METABOLISM OF THE ALBINO RAT

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The object of the present paper is to present data showing the effect of season, sex and weight upon the basal metabolism of the albino rat, and to compare the results computed as calories per kilogram per hour with those of calories per square meter per hour.

Sherwood, Savage and Hall ('33), Sherwood, Toth and Carr ('34), Sherwood and Bowers ('36), while investigating the effect of various substances upon the respiratory metabolism of the rat obtained approximately 6000 basal tests on both males and females over a period of 5 years. Out of this extensive data 1962 normal basal metabolism tests were obtained before the animals were used for experimentation. The normal tests represent data on seventy males and 100 females.

The presentation of the normal basal metabolism thus made possible seems of practical value since it may be used by future investigators for comparison with experimental data obtained during the various seasons.

## EXPERIMENTAL PROCEDURE

The animals were fed a well-balanced dry ration (dog chow)<sup>1</sup> which was supplemented with fresh whole milk and green plant material. They were kept in an environmental temperature which varied within the limits of 21°C. to 29°C., the average temperature being approximately 26°C.

<sup>1</sup> Purina brand.

The apparatus used was a modification of the Haldane open circuit type (1892) which has been successfully used for small animals such as the rat, mouse, rabbit and guinea pig. The apparatus was kept in a room of thermic neutrality (28°C.) where the basal metabolism determinations were made.

Horst, Mendel and Benedict ('34 a) found that activity increased the basal metabolism in young males per unit of surface but not per unit of body weight. The rats used in the present investigation were kept in the same type of cage the year round and were therefore not exposed to an exercise variable. The hours between 10 A.M. and 4 P.M. as shown by Horst, Mendel and Benedict ('34 b) were found to be the most desirable for metabolism experiments since the animals usually slept during the major portion of the 3-hour testing period. It was found that when slight movement occurred normal heat production was not disturbed, which is in confirmation of the report of Benedict and MacLeod ('29 a).

The animals soon learned to adjust themselves to the 18-hour fast and regained the weight lost during the beginning tests. Probably due to their inability to withstand the fasting periods, the younger rats weighing less than 100 gm., did not give the consistent results obtained in the higher weight groups.

The heat production was calculated for surface area (Diack, '30) as well as for weight. During a preliminary investigation of 6 months to find the most suitable testing period, 2-, 3-, 4- and 6-hour periods were used. The 2-hour period did not give a sufficient change in weight of the apparatus for reliable measurement. The increased activity of the rats during the 4- and 6-hour periods seemed to interfere with the normal basal metabolism. The 3-hour period was therefore used for each basal metabolism determination.

More than 3500 normal basal metabolism determinations were obtained. However, nearly one-half of these were discarded since they followed experimentation. Only data were used that were known to be normal in every respect.

Benedict and MacLeod ('29 b) considered nineteen tests covering a period of 6 months detrimental to the health of the animal and, therefore, disturbing to the basal metabolism. Many rats in this laboratory have had basal metabolism tests every other day for a period of 6 months and no ill effects were observed. The weight was kept constant and the heat production did not vary from the normal. One female was used successfully from January to January of the next year and gave normal results when compared with the control rats of the various seasons.

### RESULTS

Table 1 represents data obtained upon seventy males weighing from 100 to 420 gm. In the table are presented both the weight and surface area calculations for the sake of comparison. The actual metabolism averages for the weight groups as well as for the months are given. The number of tests for each weight group and each month are also presented.

Where the data are sufficient, as in the group of animals weighing from 141 to 260 gm., there is some indication of a seasonal variation. The heat production is slightly lower during the summer months. This picture is less pronounced in the males than in the females.

During the month of February the data are sufficient to show the effect of weight upon the basal metabolism. The surface area data show a difference of 26% in heat production between the rats weighing 100 gm. and those weighing 420 gm. The data calculated as calories per kilogram per hour for the month of February show a 42% difference in the weight range mentioned above. The tendency for a decrease in heat production as the animals increase in weight is indicated more or less for all other months.

In table 2 the data represent the metabolism to the extent of 1259 tests obtained upon 100 females ranging in weight from 100 to 420 gm. The data are presented in the same manner as shown for the males. There is no indication of a seasonal variation in the animals weighing less than 181 gm.

TABLE 1  
*Basal metabolism of the male rat*

	GRAMS								TOTAL TESTS	ACTUAL AVERAGE
	100- 140	141- 180	181- 220	221- 260	261- 300	301- 340	341- 380	381- 420		
Calories per square meter per hour										
January	49.7	41.5	37.3	43.6	39.5				83	42.2
February	46.9	45.5	39.5	39.9	39.6	42.5	36.3	34.4	127	40.9
March		43.8	40.6	33.3	37.7	37.4	34.3		87	38.6
April		40.9	37.7	37.0	34.2				78	38.2
May	43.6	39.7	32.6	37.9					57	38.2
June		38.1	37.1	36.4					49	37.2
July		39.6	36.5	31.7					16	38.9
August		37.5	35.8	37.1	39.0				29	37.7
September		37.8	39.8	42.8	40.1				13	42.0
October		39.5	43.7	40.3					27	40.7
November	38.7	38.8	38.2	39.9	37.5				56	38.6
December	41.0	37.5	39.9	31.8					81	38.4
									(703 total)	
Number tests	40	205	190	181	60	7	16	4		
Actual average	46.2	40.7	38.3	40.2	31.5	40.3	35.4	34.4		
Calories per kilogram per hour										
January	7.38	5.85	5.34	5.22	4.57				83	5.87
February	6.90	6.29	5.05	4.79	4.61	4.70	3.88	4.00	127	5.15
March		5.93	5.17	4.38	4.37	4.17	3.70		87	4.71
April		5.60	4.82	4.40	3.89				78	4.88
May	6.38	5.46	5.13	4.33					57	5.38
June		5.25	4.68	4.12					49	4.68
July		5.36	4.49	3.80					16	4.82
August		5.27	4.72	4.67	4.50				29	4.75
September		5.52	5.04	5.20	4.69				13	5.17
October		5.36	5.32	4.88					27	5.16
November	5.63	5.16	4.27	4.90	4.30				56	4.97
December	5.94	5.10	5.29	4.47					81	5.20
									(703 total)	
Number tests	40	205	190	181	60	7	16	4		
Actual average	6.82	5.58	5.08	4.86	3.64	4.47	3.80	4.00		



TABLE 2  
*Basal metabolism of the female rat*

	GRAMS								TOTAL TESTS	ACTUAL AVERAGE
	100- 140	141- 180	181- 220	221- 260	261- 300	301- 340	341- 380	381- 420		
Calories per square meter per hour										
January	42.3	39.3	39.7	38.6	36.8	36.0	38.8		157	39.1
February	41.3	40.7	40.0	31.5	39.3	35.2	30.8		209	39.1
March	40.0	39.5	40.8		35.3	37.1			196	39.2
April	39.9	40.6	43.1	38.6					177	40.9
May	44.7	40.3	38.0	41.8					102	40.5
June	37.6	38.9	36.7						21	37.6
July	41.4	39.3	37.5	39.2	39.4	35.2			41	38.3
August	38.9	42.5	38.4	37.5	37.3				106	39.0
September	45.9	41.0	43.6						52	40.7
October	41.9	41.3	43.6						58	42.2
November	37.3	38.1	38.3						52	38.0
December	42.2	39.4	38.7						87	39.8
									(1259 total)	
Number tests	117	590	369	31	101	49	2			
Actual average	41.1	40.2	39.8	37.7	36.6	35.9	34.8			
Calories per kilogram per hour										
January	6.23	5.43	5.12	4.79	4.28	3.79	4.15		157	5.07
February	6.04	5.59	5.20	3.76	4.20	3.88	3.29		209	5.06
March	5.87	5.34	5.18		3.97	4.08			196	5.11
April	5.74	5.53	5.61	4.76					177	5.47
May	6.73	5.52	4.96	5.13					102	5.54
June	5.55	5.21	4.74						21	5.05
July	6.56	5.34	4.81	4.59	4.57	3.94			41	5.00
August	5.94	5.89	4.91	4.66	4.33				106	5.02
September	6.66	5.68	5.05						52	5.37
October	6.06	5.68	5.65						58	5.68
November	5.50	5.08	4.96						52	5.18
December	6.23	5.36	5.05						87	5.44
									(1259 total)	
Number tests	117	590	369	31	101	49	2			
Actual average	6.07	5.48	5.12	4.59	4.19	3.90	3.72			

The weight group from 181 to 220 gm. demonstrates a definitely lower heat production during the summer months. However, the data are not sufficient to show this phenomenon in the larger and older animals. The females weighing 100 gm. show a 25.4% higher heat production than those weighing 380 gm. when calculated on the number of calories per square meter per hour. The calculations based on the calories per

TABLE 3  
*Basal metabolism of the rat*

MALES			FEMALES		
Weight in grams	Calories kilo/hour	Calories sq.m./hour	Weight in grams	Calories kilo/hour	Calories sq.m./hour
131	6.63	45.1	131	6.77	45.3
136	6.37	43.9	136	6.10	42.0
146	6.17	43.3	146	6.07	42.5
151	5.87	38.1	151	5.70	40.6
158	5.23	37.8	158	5.38	39.0
168	5.29	38.9	168	5.39	41.5
170	5.33	39.6	170	5.22	39.1
175	5.13	39.2	175	5.19	38.9
179	5.03	38.2	179	5.03	38.0
189	5.20	39.8	189	5.17	39.8
197	4.60	38.8	197	4.98	39.0
211	4.53	38.6	211	4.82	38.3
220	4.90	39.5	220	4.93	39.9
226	4.74	38.9	226	4.64	38.0
247	4.69	39.3	247	4.48	35.0
264	4.52	39.0	264	4.01	34.4
277	4.62	39.9	277	4.30	37.6
289	4.17	37.3	289	4.05	36.0
302	4.15	37.2	302	4.00	35.6
304	4.18	37.5	304	3.84	34.5

kilogram per hour show a 45.5% difference in the same group of animals.

The coefficient of variability shows the males 7% more variable than the females when measured in calories per square meter. This means that on the average one male differs from another male 7% more than one female differs from another female when the variability is calculated on

the basis of calories per square meter. When measured, however, in calories per kilogram the males are only 5.4% more variable than the females. The heat production measured in calories per kilogram is more variable within both sex groups

TABLE 4  
*Respiratory metabolism of the female*

	NUMBER TESTS	AVERAGE CAL./KILO PER HOUR	STANDARD DEVIATION	SIGMA DIFFERENCE	OBTAINED DIFFERENCE	CHANCES <sup>1</sup> IN 100
January	148	5.12	0.798			
February	163	5.03	0.903	0.096	0.09	83 <sup>1</sup>
March	246	5.09	0.798	0.087	0.06	76
April	216	5.56	0.580	0.064	0.47	100
May	148	5.49	0.626	0.065	0.07	85
June	39	4.99	0.607	0.110	0.50	100
July	29	4.99	0.584	0.146	0.00	50
August	105	5.28	0.632	0.124	0.29	99
September	77	5.38	0.590	0.091	0.10	86
October	54	5.29	0.538	0.099	0.09	82
November	67	5.16	0.582	0.102	0.13	89
December	100	5.52	0.632	0.095	0.36	100

<sup>1</sup> "The chances of 83 in 100" means that if 100 groups of rats were taken during January and 100 groups during February, we would find that the differences between these groups would, in 83 cases, be as indicated in the table—in 17 of the 100 there would either be no difference or the difference would be in the opposite direction. Such chances are calculated on the assumption that the errors of measurement fall on a normal distribution curve.

than is that of the surface area metabolism. This fact is indicated by the standard deviations of the two groups.

Table 3 shows a comparison between animals of equal weight. Data were available on fifty females and fifty males. An average of ten tests on each animal shows a slightly

higher heat production in the male than in the female, in the larger animals. The younger animals have approximately the same basal metabolic rate.

Table 4 presents the standard deviation for each month for the females. The reliability of the difference from month to month is given in terms of the chances in 100 of there being

TABLE 5  
*Respiratory metabolism of the male rat*

	NUMBER TESTS	AVERAGE CAL./KILO PER HOUR	STANDARD DEVIATION	SIGMA DIFFERENCE	OBTAINED DIFFERENCE	CHANCES IN 100
January	83	5.25	0.449			
February	127	4.92	0.594	0.083	0.33	100
March	87	4.77	0.611	0.082	0.15	96
April	78	4.84	0.678	0.111	0.07	74
May	57	5.18	0.655	0.112	0.34	100
June	49	4.77	0.727	0.130	0.41	100
July	16	5.30	0.619	0.253	0.53	98
August	29	5.27	0.603	0.250	0.12	56
September	13	5.08	0.418	0.140	0.21	93
October	27	5.68	0.504	0.180	0.62	100
November	56	5.22	0.710	0.190	0.46	99.2
December	81	5.14	0.578	0.140	0.57	71

a true difference in the metabolism from one month to the next. These chances were calculated on the assumption that the basal metabolism of the female albino rat followed the normal distribution curve and that adequate data were obtained on the basal metabolic rate. There is evidence that there is a true difference between the succeeding months of the year, except between June and July. Perhaps a reliable

TABLE 6  
*Respiratory metabolism of the male rat*

WEIGHT IN GRAMS	NUMBER TESTS	AVERAGE CAL./KILO PER HOUR	STANDARD DEVIATION	SIGMA DIFFERENCE	OBTAINED DIFFERENCE	CHANCES IN 100
125-175	211	5.48	0.630	0.059	0.44	100
176-225	207	5.04	0.571			
176-225	207	5.04	0.571	0.051	0.32	100
226-275	196	4.72	0.445			
226-275	196	4.72	0.445	0.068	0.01	56
276-325	53	4.71	0.441			
276-325	53	4.71	0.441	0.117	0.71	100
326-400	25	4.00	0.500			

TABLE 7  
*Respiratory metabolism of the female rat*

WEIGHT IN GRAMS	NUMBER TESTS	AVERAGE CAL./KILO PER HOUR	STANDARD DEVIATION	SIGMA DIFFERENCE	OBTAINED DIFFERENCE	CHANCES IN 100
125-175	685	5.63	0.662	0.035	0.46	100
176-225	540	5.17	0.573			
176-225	540	5.17	0.573	0.060	0.76	100
226-275	53	4.39	0.395			
226-275	53	4.39	0.395	0.069	0.39	100
276-325	94	4.00	0.414			
276-325	94	4.00	0.414	0.084	0.15	96
326-400	20	3.85	0.321			

difference would have been obtained if it had been possible to collect more data for these months.

Table 5 gives the comparable data for the male albino rat. The differences are clearly demonstrated except between July and August. An inspection of the averages reveals no definite seasonal curve. The seasonal effect is more pronounced in the females but the male basal metabolism shows greater variation.

Table 6 shows a decided decrease in metabolism as the males increase in weight except those weighing from 226 to 325 gm. Here the metabolism seems to remain rather constant until the rats reach a larger weight group where the drop is again very definite. In table 7 the females show a decided decrease for every group as the weight increases. The critical ratio also becomes smaller as the animals increase in weight.

#### DISCUSSION

The data of this investigation were obtained from animals of the age commonly used in experimental laboratories. Since a survey of the basal metabolism of the albino rat seemed necessary it was decided to produce as complete a picture as possible by using both sexes and as many different weight groups as available.

A comparison of the data of the present investigation with those of Benedict and MacLeod ('29 b) shows the value of a greater number of basal metabolism tests for such an extensive problem as that undertaken by them. There is no indication of an increase in the basal metabolism with increasing age in the rats used in the present investigation as was reported by the above-mentioned authors who used animals as old and older than our larger weight groups. The most desirable animals for basal metabolism investigation were found in the weights ranging from 181 to 300 gm.

The heat production in the control rats reported by Horst, Mendel and Benedict ('34 a) was slightly higher than that found in the present investigation. Horst, Mendel and Benedict ('34 b) found the hours 10 A.M. to 4 P.M. suitable

for measuring the heat production. In the present investigation it was noticed that the rats usually slept during the 3-hour testing period. When the animals were excessively active the tests were discarded.

Upon examination of the data of the above authors one sees a decrease in metabolism with increasing age in both males and females which is in agreement with the data of the present investigation. However, the statement is made that males have a higher heat production than females. Rat no. 3 of their data, a male weighing 137 gm., shows a heat production of 27.2 cal. per 200 gm. per 24 hours compared with rat no. 9, a female, weighing 139 gm., 30.4 cal. per 200 gm. per 24 hours. This male is younger and should therefore have a much higher heat production. Female no. 2 of the same data shows a heat production of 27.3 cal. compared with the lower heat production of 22.2 cal. of a male weighing 239 gm., the female in this case weighing 236 gm. The data seem inadequate for a comparison of sexes and the number of females, six animals, not sufficient for comparison with the males, fifty-four animals.

Horst, Mendel and Benedict ('34 b) state that rats weighing from 100 to 320 gm. have essentially the same metabolism at 30°C. when measured per unit of surface area, in reviewing the work of Houssay and Artundo ('29) but fail to mention that these authors show a heat production of 8.2 cal. per kilogram per hour in rats weighing from 100 to 139 gm. and a heat production of 5.7 cal. per kilogram per hour for rats weighing from 280 to 320 gm. This is a reduction of approximately 30% in heat production from the smaller to the larger animals, a phenomenon which is certainly worthy of mention. In the present investigation there was a decrease in heat production of approximately 26% when calculated per unit of surface area in the weight range from 100 to 420 gm. and a 45% decrease in the same weight range when measured per kilogram per hour. The metabolism of the rats mentioned by Houssay and Artundo ('29) is somewhat higher at 30°C. than that found in the present

investigation. This may have been due to the fact that our animals were kept in a room where the average yearly temperature was between 25°C. and 29°C. thereby causing a lower heat production.

Benedict and MacLeod ('29 b) show a seasonal variation in the basal metabolism of the albino rat but do not take into consideration size, sex and age effects with respect to seasonal heat production. The present investigation does not show a seasonal variation in all weight groups. It appears in animals weighing above 180 gm. but below that weight there is no indication of a seasonal influence in either sex. It may be that in young growing rats the seasonal variation is obliterated by the higher heat production and constant change in weight.

Gustafson and Benedict ('28) attempt to show a seasonal variation in the basal metabolism of young women. Unfortunately they present no data for the months of July, August and September which seem important for a complete seasonal picture.

Mitchell and Carman ('26) report a decrease in heat production with an increase in weight and age in younger animals. They show that the basal metabolism of male rats per unit of body surface area or of body weight is more variable than that of female rats. In both sexes the variability of the basal metabolism referred to body surface was less than that referred to body weight.

With so many cases available, it was thought that a comparison of the two methods used in calculating heat production would be of value. That the two methods do not measure the same thing is evident since the coefficients of correlation are 0.84 and 0.71 for the females and males respectively. These coefficients are high, but since they have been corrected for sampling errors, one would expect a coefficient of 0.90 or above if they were equally valid.

A word might be said about the meaning of coefficients of correlation. The range of the coefficient is from plus one to minus one. A coefficient of plus one indicates that the two series of measurements are so closely related that one may



be used as an indication of the other. It does not mean, however, that the one is the cause of the other. The coefficients of 0.84 and 0.71 obtained in the present investigation indicate, therefore, that the two technics used in calculating heat production do not give identical results. That is, we cannot use one to predict, with perfect reliability, the other.

The data also afford some information as to the reliability of the two technics. By perfect reliability it is meant that the technic applied on two different occasions to the same organism under the same conditions will give the same result. Consequently, the metabolic reading obtained on 2 successive days, on the same rats were correlated. Surface area measurements were as follows: For females 0.83, for males 0.79. Measurements per kilogram were 0.88 for females and 0.83 for males. If the technics were perfectly reliable, the coefficients would have been plus one. We may conclude from these coefficients that the weight technic is slightly more reliable. However, this might not have been the case if other surface area formulae had been used in the calculations.

In spite of the slightly higher reliability of the weight technic, the surface area technic may be more valid. That is, it may measure more adequately, the daily heat production.

#### CONCLUSIONS

Adult rats weighing above 180 gm. show a slight seasonal variation in basal metabolism, being somewhat lower during the summer months.

The data show a decrease of 26% in heat production from animals weighing 100 gm. to those weighing 420 gm. when measured in calories per unit of surface area. A decrease of 42% is shown for the same group when measured in calories per kilogram.

The male has a more variable heat production than the female.

The basal metabolic rate is shown to be nearly the same in young males and females, to vary during active sexual life and finally to become nearly the same as the rats approach the end of sexual life.

The metabolism calculated as calories per square meter does not give the same picture as that calculated per kilogram of body weight.

The weight technic is slightly more reliable than the surface area technic as measured by Diack's formula.

The author wishes to express his appreciation for the helpful criticism given by Dr. Walter J. Meek in the method of presenting the material of the present investigation; to Dr. J. A. E. Elyster for his criticism of the statistical methods used in presenting the data; and to Dr. M. M. White for his most generous assistance with the statistical data.

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# THE AVAILABILITY OF THE PROTEINS AND INORGANIC SALTS OF THE GREEN LEAF

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## ONE FIGURE

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## INTRODUCTION

The importance of vegetables in our diet has greatly increased in recent years due to the fact that attention has been centered upon the leafy foods as excellent sources of the 'little things in nutrition,' i.e., the vitamins and the inorganic salts. So extensive have been the investigations of these vital principles, that one probably knows much more about the composition of vegetables with respect to their content of vitamins and inorganic ions than about the amounts of digestible carbohydrate, fat and protein which they contribute in nutrition. While it is true that we do not ordinarily eat leafy vegetables as sources of energy, there are occasions when it is necessary to know very exactly the available protein, fat and carbohydrate content of these foods.

Most of the data on the composition of American food materials are based on Atwater and Bryant's famous bulletin no. 28 ('02). In this work, protein was determined by multiplying the total nitrogen of the foodstuff by a factor, 6.25.

<sup>1</sup>The data in this paper were taken from a dissertation presented by M. K. Horwitt in partial fulfillment of the requirements for the degree of doctor of philosophy, Yale University, 1935. Lack of space prevents the presentation of more detailed data than are reported here. Additional details are in the dissertation deposited in the Yale University Library.

A preliminary report of this material was presented before the American Institute of Nutrition, Washington, D. C., on March 25, 1936.

<sup>2</sup>Deceased December 9, 1935.

The inadequacy of this method has long been recognized. Not only is it true that all of the nitrogen is not protein nitrogen, but the use of the factor, 6.25, based on the fact that protein on an average contains 16% nitrogen, is often misleading. Jones ('31) has made a study of protein nitrogen and has recommended the use of different factors for different proteins.

The term 'fat' in food analysis is equivalent to the total ether extract. The ingredients of the 'ether-extract' of vegetable foods include along with the true fats and fatty acids various other substances such as lecithin, chlorophyll, xanthophyll and carotene. The pigments alone in some green leaves may account for as much as 50% of the 'ether-extract.' The inclusion of these indigestible residues as true fat may cause a large error when the caloric value of a food is determined.

The determination of total carbohydrate as ordinarily performed is probably the least accurate of all analyses conducted on foodstuffs. The reason for this lies in the fact that this proximate principle is estimated by difference, that is, the total per cent of the moisture, ash, protein and fat is subtracted from 100% and the result called carbohydrate. Within recent years, because of the importance of preparing accurate food tables for diabetic diets, several methods have been suggested by which more accurate results may be obtained.

The fact that all the mineral constituents of vegetables are not available to the animal organism is not new. Little is known, however, concerning the amounts of the different mineral constituents which are assimilated.

At the beginning of the present study of the digestibility of leafy vegetables, it was our intention to use a variety of leafy foods in an attempt to obtain comparative results. When it became apparent that the methods available for food analysis as described in the literature were totally inadequate and that most of our time would have to be spent in the development of methods, it was deemed advisable to confine our studies, for the time being, to a single complex food substance.

It was believed that a thorough investigation of one food might be more satisfactory than a study of many foods in which less time could be spent on the different components of each.

Because of its cheapness and accessibility, and because it is representative of a type of vegetable about which there has been much discussion, spinach (the leaves of the plant, *Spinacia oleracea*) was chosen as the material for this study. It contains many of the substances which make difficult the analyses of vegetable foods, as well as a high nitrogen content and a representative amount of fat, carbohydrate and mineral matter.

Preliminary experiments with samples bought daily in the New Haven markets showed that the individual samples differed greatly from each other in composition. It was evident that if methods were to be developed, a material of more constant composition would have to be obtained. After due consideration a commercially dried spinach was selected, and a sufficient supply obtained and preserved in air-tight containers.

Some idea of the constancy of the composition of this dried spinach is gained from the fact that three separate samples of the mixed material averaged  $6.45 (\pm 0.03)\%$  moisture, while the ash and nitrogen contents averaged  $19.27 (\pm 0.04)$  and  $4.41 (\pm 0.02)\%$ , respectively, the latter two figures being expressed on a moisture-free basis.

In this paper and those which follow, the inadequacy of some of the methods applied in food analysis will be pointed out. An effort will be made to demonstrate the differences between total protein, fat and carbohydrate and the available foodstuff; these discrepancies are especially striking in the case of leafy foods. Wherever possible, availability was determined by means of *in vitro* digestion experiments.

#### PROTEINS

Ever since Stutzer (1880) applied the technic of *in vitro* digestion to protein analysis numerous attempts have been

made to determine the digestibility of different foods by estimating the increase in soluble nitrogen after digestion with pepsin or trypsin, or both. Since the literature on this topic is very extensive, it would not be practical to review the field in this paper. Some of the earliest applications of peptic digestion in the study of proteolysis are discussed by Berg ('09), but for the most part the results of the different investigators are difficult to compare because of the general lack of uniformity in the digestion methods employed.

### *Experimental*

The laboratory experiments conducted on the *in vitro* digestion of the proteins of the green leaf can be classified under two main headings: a) Digestion studies in which the classical Kjeldahl nitrogen method is the criterion for the extent of digestion, and b) those in which the amino nitrogen liberated is determined.

a. Since other parts of the green leaf in addition to protein go into solution during a proteolytic digestion, it was thought that determinations of the total solids and total ash would be of value in the subsequent interpretation of the results. At first, the determination of the total solids presented a problem because of the difficulty encountered in filtering the digested mixture. After several unsuccessful trials in which Jena glass filters, Gooch crucibles and ordinary filter papers were used, it was found that the best results could be obtained with no. 40 Whatman filter paper in an ordinary filter funnel, if very slight suction was used during the filtration. By connecting a series of filter-flasks to a pump and by adjusting the extent of the vacuum to about 100 mm. of mercury, it was possible to get good suction and reduce to a minimum the chances of the filter paper tearing.

About 1 gm. of the dried spinach, accurately weighed, was transferred to a 50 cc. centrifuge tube. The digestive agent in 40 cc. of the appropriate solvent was then added to the spinach and thoroughly mixed. The solvents used were water, N/10 hydrochloric acid, and 0.5% sodium carbonate. Blank determinations were run parallel to the enzyme digestions for the full time interval of 24 hours for each. Where successive digestions were carried out, the hydrochloric acid

was filtered off at the end of 24 hours, before the digestion in the sodium carbonate was begun. To each tube, 0.2 cc. of tricresol was added as an antiseptic; then the tube was stoppered and incubated at 38°C. Several times during the day, the tubes were shaken by hand. At the end of 24 hours the separation of the liquid from the residue was begun. Each tube was centrifuged for 5 minutes at a moderate speed. Some of the residue floated on the surface, but the major portion of the digest was packed at the bottom of the tube. The liquid was carefully transferred to the weighed filter paper and filtered with the aid of slight suction. The residue in the centrifuge tube was then mixed with 40 cc. of distilled water and the process repeated until the solid had been washed a total of three times. At the end of the third washing the solid in the centrifuge tube was transferred quantitatively onto the same weighed filter paper. Air was drawn through this residue on the filter paper until it was almost dry; the filter paper plus the residue was carefully removed from the filter funnel and analyzed for total solids by drying in vacuo at 100°C. for 24 hours or more. After weighing, this dried material was incinerated, first over a free flame until thoroughly charred and then in an electric furnace at 550°C. until no carbon particles remained. The residue left after incineration was weighed and called the 'ash in residue.'

Nitrogen in the residue was determined on a different lot of spinach digestions by means of a Kjeldahl method.

Every digestion determination was carried out in duplicate.

The moisture, ash and nitrogen content of the enzymes<sup>3</sup> used were determined separately and proper correction made in each case. A summary of a typical series of digestions is given in table 1. The data in this table represent the averages of four determinations.

A preliminary experiment to determine whether the bacteria of the intestine have a digestive effect on vegetable matter was performed as follows:

Approximately 1 gm. of dried spinach was incubated with 40 cc. of an aqueous extract of human feces which had been filtered through a coarse filter paper. After 40 hours the

<sup>3</sup> The pepsin used was from the Wilson Laboratories, Chicago, Ill.; the trypsin was obtained from Fairchild Bros. & Foster, New York, N. Y. The manufacturers claimed it was eight times the strength of Pancreatin, U. S. P.

residues were washed and analyzed for solids, ash and nitrogen, by means of the technic described above. The results of this experiment are included in table 1.

Examination of table 1 shows that water alone takes out 43.7% of the total solids in the leaf, which means that almost one-half of the substance of the cell is soluble. Hydrochloric acid removes a little more than water, and sodium carbonate in turn removes slightly more than hydrochloric acid, or 52.6%. During digestion with pepsin and trypsin, each enzyme brought a little more solid into solution. The 67% figure obtained, when both pepsin and trypsin were used successively, is about 12% higher than the result of the control extraction with hydrochloric acid and sodium carbonate.

TABLE 1  
*Summary table of comparative digestions*

DIGESTIVE AGENT	SOLIDS EXTRACTED	ASH EXTRACTED	NITROGEN EXTRACTED
	%	%	%
Extraction with water	43.7 $\pm$ 0.2	83.8 $\pm$ 0.1	36.4 $\pm$ 2.8
Extraction with N/10 HCl	46.4 $\pm$ 0.3	88.3 $\pm$ 0.1	35.0 $\pm$ 1.0
Extraction with Na <sub>2</sub> CO <sub>3</sub> 0.5%	52.7 $\pm$ 0.1	82.4 $\pm$ 0.4	53.4 $\pm$ 0.2
N/10 HCl followed by Na <sub>2</sub> CO <sub>3</sub> (24 hours each)	55.1 $\pm$ 0.2	85.8 $\pm$ 0.2	44.2 $\pm$ 1.3
N/10 HCl plus 20 mg. pepsin	58.3 $\pm$ 0.2	88.7 $\pm$ 0.1	76.1 $\pm$ 0.4
Na <sub>2</sub> CO <sub>3</sub> 0.5% plus 50 mg. trypsin	61.8 $\pm$ 0.1	82.4 $\pm$ 0.4	84.5 $\pm$ 0.3
Pepsin followed by trypsin (24 hours each)	67.4 $\pm$ 0.1	88.8 $\pm$ 0.6	89.3 $\pm$ 1.2
Intestinal bacteria	46.0 $\pm$ 0.1	82.9 $\pm$ 0.4	44.8 $\pm$ 0.4

There are only slight differences between the amounts of ash extracted by the different agents.

Examination of the nitrogen data shows that less nitrogen was extracted by the hydrochloric acid than by any other solvent. This is to be expected because hydrochloric acid precipitates the protein present.

Proof that protein is extracted by water alone was obtained by acidifying a water extract of spinach; a copious precipitate formed which could be completely decolorized by washing in alcohol and acetone. This impure preparation gave protein color reactions, had a nitrogen content of 14% and could be coagulated by heat.



Sodium carbonate dissolved about 17% more nitrogen than water. This was not unexpected for both Osborne and Wake-man ('20) and Chibnall ('24) have shown that the protein of spinach is soluble in weak alkali. Pepsin alone made 76% of the nitrogen soluble in contrast to trypsin alone which brought 84.6% of the nitrogen into solution. That digestion studies of foods should be done by successive treatment with pepsin and trypsin is brought out by the fact that such treatment dissolved more nitrogen than the use of either of the enzymes individually.

The results of the experiment in which intestinal bacteria were the digestive agents show that there is not much difference between the effect of bacteria and the solvent action of pure water.

Approximately 90% of the nitrogen in spinach was made soluble by enzymatic activity. What is the nature of the other 10%?

Since chlorophyll and the cephalin-like compounds contain nitrogen and are soluble in ether, an experiment was performed to determine what percentage of the total nitrogen is in the ether extract. The results showed that about 5.67% of the total Kjeldahl-determined nitrogen is fat-soluble. The importance of this observation in food analysis, in which the nitrogen alone is the sole criterion of the protein content of the food, is self-evident.

*b. Amino nitrogen studies.* Studies of the *in vitro* digestion of proteins, in which one determines the amino nitrogen liberated, involve a technic entirely different from that just discussed.

A review of the development of methods for measuring amino nitrogen and the application of this to the study of the digestion of isolated proteins was made by Frankel in 1916. With this technic, it is possible to obtain an index of the extent of digestion of a protein beyond that stage in which the protein goes into obvious solution.

More recent investigations which apply the amino nitrogen liberation technic to the study of protein digestibility have

been made by Gabathules ('20), Greaves and Morgan ('34), Waterman and Johns ('21), Waterman and Jones ('21), Jones and Waterman ('22, '23) and Jones and Gersdorff ('33, '34).

If it is assumed that casein is a completely digestible protein, the digestibility of other proteins may be estimated by comparing the extent of their digestion *in vitro* with that of casein under similar conditions. An attempt was made to compare the protein of spinach with casein in order to test this technic.

The pepsin and trypsin used in these tests were identical with those described above. The erepsin was prepared according to a modification of the method used by Northrop and Simms ('28) as follows: Three portions of hogs' small intestine, each containing only the first 3 feet below the pyloric sphincter and obtained within a few hours after slaughter, were thoroughly ground and mixed with an equal volume of glycerol. Toluene was added and the mixture allowed to stand at room temperature for 2 days. This was then strained through cheese cloth and dialyzed in a cellophane tube against running water. After 12 hours dialysis, the mixture was filtered and the filtrate returned to a dialysis tube. After 8 hours of additional dialysis, the material was filtered and used.

The total amino nitrogen in the spinach was determined by hydrolyzing with 20% hydrochloric acid for 24 hours and analyzing the hydrolysates, after distilling off the HCl *in vacuo*, for amino nitrogen by the Van Slyke ('13) method; 5 gm. of dried spinach yielded 139.7 mg. In order to estimate how much amino nitrogen other than protein amino nitrogen was present in the dried leaf before it was hydrolyzed, 5 gm. samples of dried spinach were thoroughly extracted with 200 cc. of warm N/100 hydrochloric acid. The amino nitrogen in the extract was 14.6 mg.;<sup>4</sup> 139.7 mg. minus 14.6 mg. gives 125.1 mg. of amino nitrogen liberated from the protein of 5 gm. of dried spinach by acid hydrolysis.

Having estimated the total amount of amino nitrogen in the leaf, the amount which could be obtained by means of enzymatic hydrolysis was determined. All digestions were

<sup>4</sup>This figure does not include amino nitrogen from lecithin-like compounds which may be present in the leaf but this error is too small to affect the comparative results.

carried out in duplicate. Control experiments were run with all the reagents and enzymes in the same quantities as in the regular experiment except that no spinach was added. In each case, sufficient enzyme was used to give maximum digestion in terms of amino nitrogen liberated.

Twenty grams of spinach were mixed with 0.20 gm. of pepsin in N/10 hydrochloric acid and 2 cc. of tricresol. The mixture was brought to a volume of 400 cc. with N/10 hydrochloric acid and incubated in stoppered flasks at 37 to 38°C. At definite intervals 3 cc. samples of the mixture were removed, filtered and treated with 1 volume of sodium hydroxide to inhibit further peptic digestion. These samples were then analyzed for amino nitrogen by the micro method of Van Slyke ('13).

After 71 hours of digestion with pepsin-hydrochloric acid, 300 cc. of digest were withdrawn, made faintly alkaline with solid sodium bicarbonate and treated with 0.4 gm. of trypsin. These solutions were again incubated at 37 to 38°C. Samples were withdrawn at definite intervals, treated with one-third their volume of glacial acetic acid to inhibit further proteolysis and analyzed for amino nitrogen.

After 150 hours of digestion with trypsin, 75 cc. of the tryptic digest were treated with 25 cc. of erepsin solution and again incubated. Samples from this were analyzed as in the case of the tryptic digest.

A summary of the results is given in table 2. In this summary all the figures given have been corrected for the amino nitrogen liberated by the enzyme controls. The amino nitrogen present in the spinach leaf before digestion, namely, 2.93 mg. per gram of the dried leaf, has also been subtracted from each result.

In order to obtain data on the digestibility of casein, this protein was treated in the manner already described for the spinach leaf. For purposes of comparison the results of the digestion of 5 gm. casein by this method are included in table 2.

Other experiments conducted to test the validity of the digestion technic included a study of the effect of the activity of diastase on the proteolytic digestion of spinach. Carman, Smith, Havens and Murlin ('29) have intimated that proteolytic digestion, when conducted in the presence of diastase,

will be carried further than such a digestion in the absence of diastatic activity. That this was not true in terms of amino nitrogen was shown by duplicating the above described digestions of dried spinach and adding diastase to one series along with the other proteolytic enzymes. There was no increase in the amount of amino nitrogen liberated due to the presence of diastase.

TABLE 2

*Summary table showing per cent of total amino nitrogen<sup>1</sup> liberated from spinach and casein by pepsin, trypsin and erepsin*

PEPSIN			TRYPSIN			EREPSIN		
	Spinach	Casein		Spinach	Casein		Spinach	Casein
hours	%	%	hours	%	%	hours	%	%
1	8.3	8.6						
18.5	12.3	13.1						
27	13.1	13.3						
50	13.9	14.1						
71	16.3	14.9						
73			2	25.1	38.8			
97			26	34.7	58.2			
118			47	37.1	61.0			
143			72	37.9	63.1			
169			88	37.9	63.4			
191	20.3	17.4						
195			124	38.7	63.4			
221			150	38.7	63.4			
221.5						0.5	42.0	66.0
241						20	56.4	87.8
263						42	62.0	89.7
282						61	63.6	89.9
298						87	63.6	89.9
336			265	38.7	63.4			

<sup>1</sup> Twenty-five and 98.0 mg. total  $\text{NH}_2\text{N}$  per gram of dried spinach and casein, respectively.

### *Discussion and a suggested method for determining available nitrogen*

A comparison of the amino nitrogen liberated from the uncooked green leaf with that of casein (fig. 1) seems to indicate that the protein of spinach is not as easily digested as

casein since only about 40% of the amino nitrogen of the spinach was liberated as compared with 62% from the casein by pepsin plus trypsin. However, examination of the work of Waterman and Jones ('21) shows that there is a marked increase in the digestibility of some proteins after cooking. Therefore, any future work on this problem would have to include a study of the digestibility of the cooked vegetable since this is the form in which the food is consumed by man.

On the basis of the above results and of those reported by Frankel ('16) and Waterman and Jones ('21) one is strongly tempted to make the statement that vegetable proteins are not

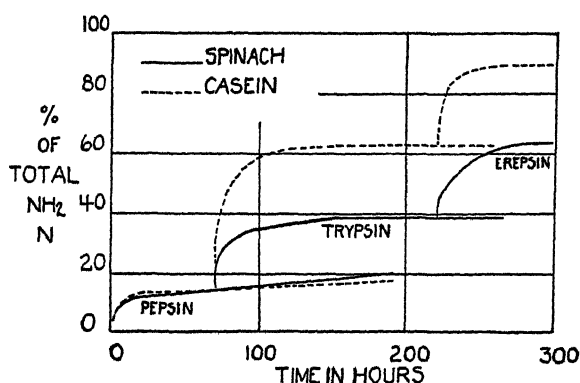


Figure 1

digested as well as casein in the alimentary tract. However, our knowledge of the mechanisms of digestion and absorption in vivo is still too limited to warrant such a conclusion. The recent studies of London ('34) showing that polypeptides are absorbed from the intestine suggest that any attempt to break down a protein completely to amino acids in order to simulate what happens in the alimentary tract, may not be the appropriate objective.

A more practical procedure, perhaps, would be to estimate with greater accuracy the available nitrogen in a food. By eliminating the fat-soluble nitrogen and the nitrate, amide and ammonia nitrogen before determining protein and amino

acid nitrogen, a more useful figure can be obtained. The following method was devised for that purpose:

Extract 2 gm. of the dried finely comminuted substance three times with 50 cc. portions of a hot alcohol-ether mixture containing 80% alcohol and 20% ether. Transfer the residue from this extraction to a Kjeldahl flask, and mix with 50 cc. water, 2 gm. of ferrous sulphate and 5 cc. of  $H_2SO_4$  (1 + 1). After the reaction has ceased, boil the mixture slowly for about 5 minutes and cool. Add water, to make a volume of about 100 cc., and about 5 gm. of magnesium oxide. Boil the mixture until almost dry, cool and carefully add 25 cc. of concentrated sulfuric acid and 1 drop selenium oxide. From this point on the procedure is the same as in the Kjeldahl determination for nitrogen. Additional potassium sulfate is not necessary because the magnesium sulfate formed is equally efficient in raising the boiling point of the mixture.

The application of this method to dried spinach gave 3.85% available nitrogen as compared with 4.41% total nitrogen in the dried tissue.

#### AVAILABILITY OF THE INORGANIC NUTRIENTS

The fact that all of the so-called ash of a foodstuff is not completely utilized by the body is now well recognized. A considerable portion of the inorganic compounds of the normal diet fails to dissolve during its passage through the alimentary tract and is therefore not absorbed. Because of their obvious importance, calcium, phosphorus and iron compounds have received most of the attention devoted to the availability of the inorganic substances.

No attempt will be made to review the extensive literature on the availability of the inorganic constituents of the diet. Typical papers in the field are those of Fincke and Sherman ('35), Sherman, Elvehjem and Hart ('34) and Blatherwick and Long ('22) on the utilization of calcium, iron and phosphorus, respectively.

*Experimental*

In the hope that application of in vitro digestion to the study of the availability of inorganic salts might prove informative, several experiments were conducted in which dried spinach was digested with enzymes. The amount of calcium, phosphorus, iron (and in a few cases the amount of chlorine) made soluble by this treatment was determined. The results of these experiments were compared with those yielded by chemical analyses for total calcium, phosphorus and iron.

*Chemical analyses for total calcium, phosphorus and iron.* About 5 gm. of dried spinach were weighed into a platinum dish and mixed with 15 cc. of a solution of 5% pure sodium carbonate. This was evaporated to dryness and carefully charred over a free flame until no more fumes were given off. The ashing procedure was completed in an electric furnace at a temperature below 550°C. for 8 hours. Particles of carbon may remain but they do not interfere with the subsequent determination. The ashed material was then extracted, first with hot water and then with hot nitric acid (1 + 4), filtered and brought to a volume of 100 cc.

Aliquots of this filtrate were analyzed for chloride by the method described by Whitehorn ('21), for calcium by Clark and Collip ('25) techniques and for phosphorus by the gravimetric procedure described in the "Official and Tentative Methods of Analysis" (A. O. A. C., '30, p. 15). In order to determine iron, 25 cc. of the filtrate were mixed with 5 cc. of concentrated hydrochloric acid and evaporated to dryness on a hot-plate to drive off the nitrate. The dried material was dissolved in a solution of weak sulphuric acid (1 + 25), heated almost to boiling, and the copper precipitated by saturating the solution with hydrogen sulphide. The cooled solution was filtered and the iron in the filtrate oxidized to the ferric state by adding 1 cc. of bromine water. The excess bromine was removed by boiling the solution for 3 minutes. The removal of bromine was greatly accelerated by having a small glass bead present in the boiling solution. This solution was then cooled, saturated with carbon dioxide, mixed with 3 cc. of a 30% solution of potassium thiocyanate and titrated with approximately N/1000 titanium sulphate in an atmosphere of carbon dioxide.

The amounts in per cent of calcium, phosphorus, iron and chloride in the dried spinach were, respectively, 1.016 ( $\pm 0.006$ ), 0.886 ( $\pm 0.002$ ), 0.0541 ( $\pm 0.0002$ ) and 1.267 ( $\pm 0.004$ ). These figures are averages of three determinations.

*Application of in vitro digestions to determine the availability of the inorganic constituents of spinach.* The procedure applied here was the same as that which was used to determine loss of total solids, above described in the discussion of protein digestion.

TABLE 3

*Availability of the inorganic constituents of spinach as estimated by in vitro digestion experiments*

DIGESTIVE AGENT	AMOUNT EXTRACTED			
	Chloride	Calcium	Phosphorus	Iron
Water	% 99.5	% 8.6	% 96.6	% 9.0
N/10 HCl		29.1	80.6	18.6
0.5% Na <sub>2</sub> CO <sub>3</sub>	99.3	12.5	90.4	25.0
N/10 HCl followed by 0.5% Na <sub>2</sub> CO <sub>3</sub>		30.0	89.0	24.9
N/10 HCl plus 20 mg. pepsin		30.5	82.5	40.0
0.5% Na <sub>2</sub> CO <sub>3</sub> plus 50 mg. trypsin	100.0	12.5	90.0	24.0
Pepsin followed by trypsin		30.2	89.9	40.6

The filtrates were mixed with 0.5 gm. of sodium carbonate and evaporated to dryness. The dry material was carefully charred, placed in a cold oven which was then brought to a temperature of 550°C. and kept there for about 8 hours. The resulting ash was extracted first with hot water and then with hot dilute nitric acid, filtered and brought to a volume of 50 cc. Aliquots of this were used to determine calcium by the method of Clark and Collip ('25). In those digestions in which hydrochloric acid had not been used, chlorine was also determined. A portion of the filtrate was freed of nitrates and analyzed for phosphorus by the method of Fiske and Subbarow ('25), and for iron by means of the titanium titration described above.

The results of these analyses, expressed in terms of percentage of total material extracted, are given in table 3.



## DISCUSSION

An examination of the results in table 3 brings out some interesting correlations between the availabilities of calcium, phosphorus and iron in vegetables as reported in the literature, and their solubilities after in vitro digestion.

The chloride of the leaf is, of course, completely soluble. In fact, some methods of feed analysis determine the total chloride present by extracting the material to be analyzed with hot water (A. O. A. C., '30).

That the phosphorus in vegetables is readily available has been championed by Blatherwick and Long ('22) and others. It is therefore not surprising to observe that nearly all of the phosphorus of spinach is extracted by water alone. Only about 30% of the total calcium present could be made soluble after treatment with N/10 hydrochloric acid, and there was no significant increase in calcium solubility when the leaf was digested with pepsin. This may serve to explain the observation of Sherman and Hawley ('22) that the calcium of vegetables is not as available as the calcium of milk. More recently Fincke and Sherman ('35) have demonstrated by means of biological tests that the calcium of spinach is poorly utilized.

Sherman, Elvehjem and Hart's ('34) method of determining iron availability by extracting the material with 10% acetic acid and then estimating the amount of iron in solution by Hill's bipyridine method ('30) gave results indicating that only about 20% of the iron in spinach was available. The data in table 3, showing that more iron was rendered soluble by hydrochloric acid plus pepsin than by hydrochloric acid alone, may be taken as indicating that more than 20% of the iron in spinach is available.

## SUMMARY

A study of the known in vitro digestion technics failed to produce a practical method that might be used to determine the utilizable nitrogen in the green leaf. The results obtained, however, suggested that a closer approximation to the available nitrogen could be obtained by eliminating the fat soluble

nitrogen, by means of extraction with an alcohol-ether mixture, and by boiling off the nitrate, ammonia and amide nitrogen from the substance to be analyzed before determining protein and amino acid nitrogen. On this basis a simple procedure for estimating the available nitrogen present has been devised.

An investigation of the digestion of spinach by successive treatment with pepsin, trypsin and erepsin was made. The results obtained when the entire leaf was digested are similar to those obtained with pure proteins. This indicates that with certain materials, at least, it is possible to perform exact proteolytic digestions without necessarily isolating the protein.

The application of in vitro digestions to determine the availabilities of the calcium, iron, chloride and phosphorus in spinach confirmed the view advanced in the literature that the calcium and iron in spinach is only partly available. Enzymatic digestion brought about 30% of the calcium and 40% of the iron into solution. Chloride and phosphorus were readily dissolved in water alone.

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# THE AVAILABILITY OF THE CARBOHYDRATES AND FATS OF THE GREEN LEAF TOGETHER WITH SOME OBSERVATIONS ON CRUDE FIBER

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## CARBOHYDRATE

The need for more precise knowledge of the carbohydrate content of food has been apparent in recent years because of the necessity of preparing accurate food tables for diabetic diets. In 1920 Olmstead published a method for determining the availability of carbohydrate in vegetables by digesting with taka-diastrase and then following the enzyme digestion with an acid hydrolysis in order to convert the maltose to glucose. This procedure was used on vegetables by O'Reilly and McCabe ('21) and Bell, Long and Hill ('25). Modifications of this method were introduced by Morgan, Strauch and Blume ('29) in their work on the almond carbohydrate and by Adolph and Kao ('34) who studied the carbohydrate of the soy bean.

Another taka-diastrase method that has been described is that of Thomas ('24) who used this enzyme preparation without a secondary hydrolysis. He claimed that under the conditions of his method the ratio of glucose to maltose formed

<sup>1</sup> The data in this paper were taken from a dissertation presented by M. K. Horwitt in partial fulfillment of the requirements for the degree of doctor of philosophy, Yale University, 1935. Lack of space prevents the presentation of more detailed data than are reported here. Additional details are in the dissertation deposited in the Yale University library.

A preliminary report of this material was presented before the American Institute of Nutrition, Washington, D. C., on March 25, 1936.

<sup>2</sup> Deceased December 9, 1935.

was approximately constant, and that if he regarded the ratio as 2.0, the error was small providing the total reduction was determined by the picric acid reduction method. Widdowson ('31) used a taka-diastrase digestion of starch and estimated the maltose and glucose formed by a modification of the Hagedorn-Jensen ('23) method.

The most extensive examination to date of the available carbohydrate in foods is that made by McCance and Laurence ('29). The total available carbohydrate of a large number of fruits and vegetables was estimated by hydrolyzing each food with hydrochloric acid and then determining the total reducing sugar. A correction was made for any pentosans which might have been hydrolyzed. More recently Widdowson and McCance ('35) reported a study on the available carbohydrate of a few fruits in which the glucose, fructose, sucrose and starch were determined individually. The sum total of these was called the available carbohydrate. In their paper they admitted that the hydrochloric acid hydrolysis used by McCance and Laurence ('29) destroyed 50% of the fructose.

Every carbohydrate found in nature may be found as a component of our food. Street and Bailey's ('15) investigation of the soy bean is an excellent illustration of the amount of work which has to be done in order to estimate the carbohydrate content of a food. These investigators determined pentosans, galactans, organic acids, invert sugar, sucrose, raffinose, starch, cellulose, dextrin and waxes. A similar study was performed on the navy bean by Petersen and Churchill ('21).

The digestibility of these various components has been a major problem in nutrition for many years. McCance has presented an excellent summary of the food value of vegetable carbohydrates (McCance and Laurence, '29).

Progress in the chemistry of the carbohydrates of foods has advanced slowly not for lack of efforts in this field, but rather because of the absence of relatively simple methods. Many investigators have carefully fractionated the carbohydrate portion of a few foods into the various components,

but each such attempt has constituted a complex problem in itself and the technic used has been too involved to find favor in the routine analyses of foods.

Since no one of the existing methods was entirely satisfactory, an attempt was made to devise a simple enzymatic procedure which, nevertheless, would not sacrifice accuracy for the sake of simplicity.

*Choice of enzymes.* An investigation of the literature showed that both diastase and taka-diastase had been used rather indiscriminately in attempts to determine digestible carbohydrates. In 1925, R. Kuhn published an investigation on the nature of the amylases in pancreatic juice, in malt diastase and in taka-diastase. He showed that both pancreatic amylase and the amylase in taka-diastase hydrolyzed starch to alpha maltose, whereas malt amylase hydrolyzed starch to beta maltose. From this one can conclude that the type of hydrolysis that taka-diastase catalyzes is more like that which occurs in the animal organism than that which is brought about by malt diastase.

The majority of taka-diastase preparations appearing on the market contain large amounts of lactose which is used as a solid diluent to bring the enzyme mixture to a standard strength. For exact work it is better to obtain a preparation that is free from extraneous reducing sugars. After trial and error, a particular commercial preparation of taka-diastase<sup>3</sup> was chosen, which will liquefy 2700 times its weight of starch in 10 minutes. The samples of this enzyme preparation which were used in the following experiments had very little, if any, reducing sugar present.

*Choice of method for determining 'glucose.'* Six different sugar methods were thoroughly tested before any one was chosen. Two of these were colorimetric, namely, Dastur and Samant's ('33) modification of the Folin-Wu procedure and Benedict's ('31) colorimetric copper method. Dastur and Samant added salts to each standard glucose solution so that the concentration of the ions in the standard was equal to

<sup>3</sup> Clarase 900% obtained from the Takamine Laboratories, Inc., Clifton, N. J.

that in the unknown. The theory behind this modification is excellent, but in actual practice this method proves too complex. Benedict's method uses a reagent devised to be only faintly alkaline in reaction. This modification works very well with blood filtrates but, when applied to the strongly buffered lead-clarified filtrates, the alkali is probably not strong enough. The same criticism can probably be made of the methods of Somogyi ('26) and of Hagedorn and Jensen (Hanes, '29), if these methods are to be applied to green leaves. Benedict's ('11) copper titration method was also used. The chief objection to this titration was the difficulty in obtaining good end points with dilute sugar solutions.

The method finally chosen was the Eynon and Lane ('23) titration. The methylene-blue end point is good; the method found to yield satisfactory reproducible results. Furthermore, the alkali used is sodium hydroxide, so that the buffering activity of the sugar filtrate has less influence than in the various methods that have been adapted to blood analyses. Eynon and Lane ('31) have suggested a modification of their method in which, by adding known amounts of sugar to the Fehling's solution before titrating, they are able to determine as little as 0.01% of sugar with desired exactness. Their method has been adopted as 'tentatively official' by the Association of Official Agricultural Chemists (A. O. A. C., '30).

Recovery experiments using this technic gave results within an error of 0.4%.

*The active maltase in the taka-diastase preparation.* That taka-diastase will convert starch to a mixture of maltose and glucose has been an accepted fact. According to the method most commonly used in sugar analyses, the hydrolysis of maltose to glucose is completed by heating with 2.5% hydrochloric acid at 100°C. for 2½ hours. In order to check this technic several determinations were made in which the reducing power of the clarified digest was estimated before and after the hydrochloric acid hydrolysis. It was found that in every case the acid hydrolyzed solution gave results that were 3 to 4% less than the digest which had not been hydrolyzed and which supposedly contained a large percentage of maltose.



A review of the literature offered two possible explanations of this phenomenon. In the first place, Davis and Daish ('13) had shown that it is impossible to convert quantitatively maltose to glucose by acid hydrolysis; the effects of variation of temperature, strength of acid and maltose concentrations were studied, but nevertheless, a destruction of a considerable amount of sugar occurred in every trial. Secondly, on comparing the amount of starch present in our digests with the amounts employed by other investigators, it was noticed that we were digesting relatively less polysaccharide than that hydrolyzed in other comparable studies. This suggested that diminution of the concentration of the substrate increases the

TABLE 1

*Hydrolysis of maltose to glucose by Taka-diastase. (Maltose in amount stated, 0.025 gm. taka-diastase 15 cc. of buffer at pH 4.6 made to 100 cc. and incubated at 38°C. for 48 hours with 1 cc. of toluene)*

MALTOSE HYDRATE	REDUCING POWER OF DIGEST EXPRESSED AS GLUCOSE	'GLUCOSE'/MALTOSE
<i>gm.</i>	<i>gm.</i>	%
1.980	1.690	85.3
0.990	0.891	90.0
0.495	0.473	95.5
0.247	0.246	99.6
0.1485	0.1480	99.7
0.0990	0.0994	100.4

extent of hydrolysis of maltose by maltase. When maltase is allowed to act on a concentrated solution of glucose, maltose is formed; when the concentration of the glucose is decreased, the amount of maltose thus synthesized is decreased.

A search for studies of the effect of taka-diastase on dilute starch solutions revealed the note by A. C. Hill ('02) in which is mentioned the fact that commercial taka-diastase completely hydrolyzes dilute starch solutions to glucose.

In order to test this maltase activity, solutions containing different concentrations of purified maltose hydrate were digested with 25 mg. of the special taka-diastase preparation for 48 hours as described in table 1.

At the end of the digestion the solutions were carefully analyzed for reducing power by the Lane-Eynon titration. The results expressed as glucose are given in table 1. It is evident that the smaller the concentration of maltose used, the greater was the extent of hydrolysis. Maximum hydrolysis is obtained when the concentration of maltose hydrate is below 0.250 gm. per 100 cc. The fact that Davis and Daish ('14) reported a yield of about 80% maltose and 20% glucose after digesting 2 gm. of starch with taka-diastrase gives these data added significance.

The results of this experiment explain why no additional glucose was obtained after treating the leaf digest with acid. Little maltose, if any, was present; it had apparently all been converted to glucose.

This information should greatly simplify any future analysis of starch or starch product.

Recovery experiments on three 220 mg. samples of thoroughly dried starch, which were boiled for 15 minutes in 50 cc. of water, cooled, and digested with 25 mg. of the taka-diastrase in a total of 100 cc. at 38°C. for 48 hours, averaged 99.6 ( $\pm 0.8$ )% recovery. The taka-diastrase was added in two equal portions at 24-hour intervals. One-half cubic centimeters of toluene was used as an antiseptic and the pH was adjusted to 4.6 with acetic acid.

*Modification of method to enable a correction for pentoses.* That free pentoses are present in the leaf in small amounts has been shown by Davis and Sawyer ('14) who fermented the maltose and glucose present with yeast and interpreted the residual reducing material as pentose. Any digestion with taka-diastrase of a material containing hemicellulose will bring about hydrolysis of some of this carbohydrate to pentose because of the presence of a cytase in taka-diastrase. Since pentose is a reducing sugar a correction must be made. Fortunately, pentose is not fermented by yeast and the available monosaccharides found in vegetable foods, glucose and fructose, are completely destroyed by yeast zymase. Whatever sugar remains after digestion of a mixture by yeast may

properly be considered as non-available. Therefore, a part of each clarified digest is treated with fresh, thoroughly washed, starch-free yeast in order to estimate the non-fermentable sugars. The total sugar obtained after digestion minus the non-fermentable sugars is taken as the available carbohydrate in the food.

*Sucrose hydrolysis by invertase.* The disaccharide, sucrose, is one of the constituents of the plant cell and any method which determines available carbohydrate must take account of this sugar. The enzyme, invertase, which quantitatively converts sucrose to fructose and glucose, has in recent years been studied more thoroughly than any other carbohydrase. Furthermore, active preparations of invertase are now available on the market.<sup>4</sup> The inversion of sucrose is adequately treated in the Official and Tentative Methods of Analysis (A. O. A. C., '30) and need not be discussed further.

*Amounts of enzyme and duration of digestion.* Fortunately for the method to be proposed, the reducing power of invert sugar and glucose are so nearly alike when determined by the Lane-Eynon methylene-blue titration that for practical purposes the difference can be ignored. The amount of sucrose present in the average vegetable is small compared to the quantity of glucose-yielding carbohydrates and the error is therefore well within the limits of the digestion error if all the sugar is expressed as glucose.

Two other variables, the amount of enzyme to be used and the duration of the digestion, had to be studied before the method could be considered adequate. In the preliminary experiments, 25 mg. of the special taka-diastrase had been considered sufficient to complete the hydrolysis of starch in 40 hours; later 50 mg. were used in order to increase the margin of safety. In an effort to learn whether there would be any advantage in adding the 50 mg. in two or three lots, 24 hours apart, instead of adding it all at once, all three of these procedures were tested. The results of these experiments are given in table 2. A perusal of the figures in this

<sup>4</sup> Supplied by Wallerstein Laboratories, 171 Madison Ave., New York, N. Y.

table shows that it is best to add the special taka-diastase in two lots, 24 hours apart, and that there is no advantage in adding the enzyme preparation in three lots since the maximum availability is obtained after 50 hours. In actual practice it is not advisable to digest longer than 44 hours since Davis and Daish ('14) have shown that a loss of sugar may occur if starch is digested by taka-diastase for longer than 44 hours.

TABLE 2  
*Determination of available carbohydrate in dried spinach*

		AMOUNT OF REDUCING MATERIAL RELEASED BY TAKA-DIASTASE AND INVERTASE	AMOUNT NOT FERMENTED BY YEAST	AVAILABILITY EXPRESSED AS GLUCOSE
	hours	%	%	%
(A)	16	10.18	2.24	7.94
Adding all of enzyme in one lot at the start	32	10.18	2.24	7.94
	45	10.58	2.34	8.24
	55	10.18	2.14	8.04
(B)	40	11.49	3.10	8.39
Adding taka-diastase in two lots of 0.025 gm. each 24 hours apart	50	11.61	3.21	8.40
	70	11.55	3.21	8.34
	70	11.64	3.21	8.34
(C)				
Adding taka-diastase in three lots of 17 mg. each, 20 hours apart	70	11.75	3.32	8.43
	70	11.73	3.32	8.41

*The method in detail.* An amount of dried, thoroughly powdered material (3 gm. spinach) containing less than 0.250 gm. of available carbohydrate is mixed with approximately 180 cc. of water and heated at 100°C. for 1 hour to gelatinize the starch. This is cooled to 40°C. and acidified with 5% acetic acid (about 12 drops) to pH 4.6. Twenty-five milligrams of the special taka-diastase and 1 cc. of toluene are added to the mixture which is then incubated at 38°C. At the end of 24 hours, an additional 25 mg. of taka-diastase together with 5 cc. of a solution of invertase 'free from melibiase' are added to the mixture. After 20 more hours in the incubator, 5 cc. of a saturated solution of neutral lead acetate are added to the digest which is then cooled to room temperature and diluted to a volume of 200 cc.

The mixture is filtered and a drop of methyl red added. Dry disodium phosphate is added, a little at a time, until the methyl red has lost its red color. Dry sodium carbonate is added until mixture is brought to pH 7,<sup>5</sup> after which it is filtered. This filtrate is divided into two parts; the first is analyzed by the Lane-Eynon titration for total reducing sugars, the second is fermented with yeast to give the non-fermentable sugars. The procedures are described below.

Seventy-five cubic centimeters of the clarified filtrate are treated with about 3 gm. of starch-free brewers' yeast which has been thoroughly washed by centrifuging three times with distilled water. This mixture is allowed to stand for  $\frac{1}{2}$  hour at room temperature after which time it is heated to 100°C., cooled, made to 100 cc., filtered and analyzed for reducing sugar by the Lane-Eynon titration. The calculated concentration of the non-fermentable sugars is subtracted from the total reducing sugar to give the available carbohydrate in the sample.

#### CRUDE FIBER

According to the Weende method (Henneberg, 1864) crude fiber is determined by boiling the dried food first with dilute acids and then with dilute alkali under strictly defined conditions of concentration and time, and then washing the undissolved residue with alcohol and ether. Henneberg was fully aware of the deficiencies of this method but claimed that its adaption would give an approximate determination of the cell residue. It is still used to determine crude fiber (A. O. A. C., '30).

In 1933, Remy described a biological method in which he used enzymes to separate cellulose, lignin and hemicellulose from starch, protein and fat. Remy compared the total of the cellulose, lignin and hemicellulose obtained as residues in enzymatic digestion with values obtained by the Weende method and concluded that about 50% of the indigestible residue is made soluble during the hydrolysis with acid and alkali. This new technic was a decided improvement over anything which had been suggested before.

<sup>5</sup> Nitrazine-yellow paper is a useful indicator in this range. It was described by Wenker ('34).

Williams and Olmstead ('35) recently published a modification of Remy's technic in which they separated the components of the indigestible residue into three fractions. That part not digested by enzymes which is not hydrolyzed by strong sulphuric acid is called lignin. The fraction made soluble by the sulphuric acid is analyzed for total reducing power. That part of the latter which is not fermentable is calculated as hemicellulose, inasmuch as hemicelluloses are converted to pentoses by acids. The remaining reducing power is calculated and interpreted as cellulose since cellulose is hydrolyzed to glucose by acids.

A discussion of other methods, intended as improvements on the old Weende technic, is given in the paper by Williams and Olmstead ('35).

*A comparison of 'crude fiber' results obtained by enzymatic methods with those obtained with the so-called official method.* The enzymatic digestion procedure used was similar to that described by Remy ('33) except that trypsin<sup>6</sup> was substituted for pancreatin and the special taka-diastrase described above was used instead of the diastase used by Remy.

Three grams of dried spinach were incubated with 0.5 gm. of pepsin in 500 cc. of N/10 hydrochloric acid for 48 hours. The mixture was then neutralized to pH 7 with sodium hydroxide, brought to pH 4.5 with hydrochloric acid and treated with 0.1 gm. of taka-diastrase for 48 hours. At the end of this time the digest was filtered, the residue returned to the digestion flask and treated with 500 cc. of a faintly alkaline solution containing an extract of 0.5 gm. of trypsin. This was allowed to incubate for 4 days, toluene being added daily. The residue from this digest was washed with water until the filtrate no longer gave a test for chloride, then with alcohol and ether, and dried at 110°C. to constant weight.

The data in table 3 show that the enzymatic treatment yields residues that are more than three times as large as the successive treatment with acid and alkali characteristic of the conventional crude fiber method. This is not surprising since it has long been known that much of the indigestible

<sup>6</sup> Obtained from Fairchild Bros. & Foster, New York, N. Y.

carbohydrate is dissolved by acid hydrolysis and therefore would not be in the residue. These data constitute further examples of the deficiencies of the accepted technics of food analysis.

# THE ETHER-SOLUBLE FRACTION

Any investigation into the methods of food analysis would be incomplete if some attention were not given to the much neglected ether-soluble fraction of the food. The material commonly extracted from vegetable products and called 'crude fat,' 'ether extract,' or simply 'fat' is a mixture of several substances. One obtains not only true fats, that is, glycerides of fatty acids, but also lecithins, waxes, alkaloids,

TABLE 3

*Comparison of biochemical method of obtaining crude fiber with the method of A. O. A. C. ('30)*

	DRY SPINACH	CRUDE FIBER	CRUDE FIBER
	gm.	gm.	%
Method of A. O. A. C.	2.000	0.1672	8.36
	2.000	0.1701	8.50
	2.000	0.1690	8.45
Enzymatic method	3.000	0.8412	28.0
	3.000	0.8400	28.0

sterols, chlorophyll, xanthophyll and carotene. The method in which the ether extract is designated as the fat of a food dates back to early investigations of the Weende Experiment Station by Henneberg and has not been changed to any great extent since.

*Digestibility of components of the 'ether extract.'* The investigations of Langworthy and Holmes ('15, '17 a, '17 b) and Holmes ('18 a, '18 b, '19) on the availability of many different fats definitely showed that practically all true fats, animal or vegetable, are digested and assimilated by man. Less is known about the nutritive value of the other components of the ether extract. However, the results of recent experiments are enlightening to the extent that it is now possible to draw

tentative conclusions concerning the digestibility of these fat-soluble substances. Since lecithins are readily hydrolyzed by the digestive lipases (Sinclair, '34), these compounds can be considered completely available to the animal body.

Fischer and Hendschel ('33) studied the biological breakdown of chlorophyll in human beings. Experiments with a diet consisting mainly of spinach showed that chlorophyll was but slightly changed in the digestive tract. Magnesium and phytol are hydrolyzed from the chlorophyll molecule, but the main structure of the molecule, which is similar to that of hematin, is practically unaltered. The phylloerythrin formed may be partially absorbed but it is excreted in the bile.

That carotenoid compounds can be absorbed is proved by the phenomenon of carotinemia (Hess and Myers, '19). It is doubtful, however, whether these substances are available for energy metabolism because carotenes are very resistant to biological oxidation (Kuhn and Livada, '33). Salomon ('33) reported that the absorption bands of carotene and xanthophyll were found in fecal extracts even after 9 days of a diet devoid of these pigments. This may be taken to mean that those carotenoids which find their way into the blood stream are slowly excreted by way of the bile.

Recent studies on the metabolism of sterols indicate that the members of this class of compounds, though readily absorbed in many cases, are excreted in their original or only slightly changed state. On the basis of the work reported to date, one cannot consider the sterols as potential sources of energy.

It might be said, therefore, that the only parts of the ether-soluble fraction which are available as food are the fatty acids or compounds of the fatty acids. This should not be far from the truth, and for practical purposes of food analysis, a method which would separate the fatty acid compounds from the other components of the 'ether extract' might prove useful.



### *Experimental*

Assuming that the total fatty acids present in the saponified alcohol-ether extract are an index of the availability of the fraction, the extract was saponified and an attempt made to isolate the fatty acids from the resulting mixture. The results were very discouraging because the solubilities in ether of chlorophyll and of fatty acids are so similar that it was difficult to isolate the fatty acids. For this reason experiments depending on the use of a saponification were discontinued for the time being.

In the hope that a successful method of determining the availability of the fat-soluble fraction might be developed if more were known about the lipases, an investigation with this type of enzyme was begun. The failure of all experiments of this type led to discontinuance of this line of investigation.

At this stage of the study, data on the solubilities of the chlorophylls were examined in the hope that one could separate these pigments from the fatty acids by the choice of suitable solvents. A sentence in a paper by Willstätter ('15, p. 334) proved particularly interesting. "When chlorophyll has reached a certain degree of purity, it is still easily soluble in alcohol containing petroleum ether but, surprisingly, no longer soluble in pure petroleum ether." This unexpected observation proved a great aid to Willstätter in his isolation of chlorophyll. It likewise proved helpful in the present study in separating the fatty acids from chlorophyll in a saponified alcohol-ether extract.

*Determination of total fatty acids.* The method finally developed is a modification of the Kumagawa and Suto ('08) saponification technic. The following procedure works satisfactorily when applied to the green leaf:

A 10 gm. quantity of the dried, powdered leaf (dried in an atmosphere of carbon dioxide to prevent oxidation of fats) is placed in a 200 cc. Erlenmeyer flask and boiled with 100 cc. of an alcohol-ether (4 + 1) mixture on a hot plate for 10 minutes. The blue-black supernatant fluid is decanted into a filter paper and the filtrate collected in a 250 cc. beaker. The residue is extracted for 5 minutes with 75 cc. of the boiling alcohol-ether mixture and twice again with 25 cc. portions.

The total filtrate, which now contains all the lipids and nearly all of the pigments, is evaporated to a volume of about 100 cc. To this solution 20 cc. of approximately 10 N potassium hydroxide are added. During saponification the mixture is heated on a water bath in an open beaker until the volume has been reduced to about 30 cc. At this point, 30 cc. of water are added and the saponification continued until the total time elapsed on the water bath is 1 hour.

The total saponified mixture, which should not have more than 20% alcohol, is acidified with hydrochloric acid. A large excess of hydrochloric acid should be avoided. A strongly acidified solution would cause the formation of chlorophyll products which are more soluble in petroleum ether (Willstätter, '15). The acidified mixture is transferred to a decantation flask and extracted with petroleum ether, twice with 50 cc. and twice with 30 cc. This extract is filtered and evaporated to a volume of 50 cc. It now contains all the fatty acid, the non-saponifiable lipids, some xanthophyll (which is not very soluble in petroleum ether), most of the carotene and traces of chlorophyll products. Most of the chlorophyll products have been removed; the remaining traces are separated out in the subsequent extractions.<sup>7</sup>

The petroleum ether extract which has been evaporated to 50 cc. is extracted with 50 cc. of N/10 potassium hydroxide in 50% alcohol. This in turn is twice extracted with 25 cc. of petroleum ether. To make certain that none of the fatty acids remain with the non-saponifiable substances the petroleum ether fractions are combined and extracted with 25 cc. of the alcoholic potassium hydroxide solution. The xanthophyll and most of the carotene have been left behind in the petroleum ether fraction with the non-saponifiable lipids and the alcoholic potassium hydroxide solution is now ready for the final extraction.

The combined potassium hydroxide fractions are acidified with hydrochloric acid, using a trace of phenolphthalein, and extracted with 50 cc., 25 cc. and 20 cc. portions of warm petroleum ether. The petroleum ether extract of the fatty acids is evaporated in a tared dish, dried at 100°C. for 30 minutes and weighed.

<sup>7</sup> The phytychlorins and phytyrhodins when pure are insoluble in petroleum ether (Willstätter, '15); therefore, these compounds become less soluble, as one proceeds with the separation.

The total solids obtained by the above technic should contain practically all of the fatty acid and only minute traces of the pigments of the alcohol-ether extract.

The saponification technics suggested by Liebermann (1898) and Kumagawa and Suto ('08) involve a direct saponification of the tissues; they do not extract the total lipids before saponification. Several experiments with dried spinach showed that it was not possible to obtain results by saponifying vegetable tissue directly, probably because of the resistance offered by the cellulose structures of the plant. The alcohol-ether mixture is particularly efficient in reaching all parts of the cell and for this reason an extraction of the lipids with this solvent before saponification is to be preferred.

*Application of method.* Ten grams of powdered spinach (9.355 gm. of moisture-free material) were weighed into a 200 cc. Erlenmeyer flask, extracted with alcohol-ether and the total fatty acids present determined by the modified saponification technic described above. The average of three determinations showed that  $0.210 \pm 0.002$  gm. of fatty acids were present in the spinach used or  $2.23 \pm 0.02\%$ . Since the ether-soluble fraction as determined by a standard Soxhlet method (Leach, '20) gives an average of 5.44%, it is evident that the fatty acids account for only 41% of the ether extract and the remaining 59% are unsaponifiable matter, pigments and some glycerol.

Speer, Wise and Hart ('29) have shown that a large percentage of the total fatty acids of the spinach leaf is present as free fatty acids. For this reason it is not a simple matter to estimate the amount of true fat (glyceride esters of fatty acids) present in a vegetable substance. However, in order to facilitate dietetic calculations, it would probably not be far from the truth if each gram of fatty acid obtained by the above method was evaluated as being equivalent to 9.5 calories of energy. The use of this tentative figure would be far more accurate than to continue the prevailing custom of assigning the value of 9.0 calories to each gram of the ether-extract.

## DISCUSSION

All the methods mentioned above have been devised with the idea that they be applied not only to the green leaf but to foods and feeds in general. It is quite evident that if we are to determine the true caloric content of many of our foods we shall have to change our methods of analysis. Table 4 illustrates the differences obtained when the methods used in Atwater and Bryant's Bulletin no. 28 and the technic referred to in this paper and that of Horwitt, Cowgill and Mendel ('36) are applied to the same lot of spinach.

TABLE 4  
*Comparison of results of old and newer methods*

	BULLETIN NO. 28	NEW
	%	%
Ash	19.3	
Crude fiber	8.4	28.0
Proteins <sup>1</sup>	27.6	24.0
Fat	5.5	2.2
Carbohydrate	49.2	8.4
Calories per 100 gm.	356.0	139.0

<sup>1</sup> N  $\times$  6.25.

It is appreciated that the green leaf is not fed to man because of its caloric content and that it affords an exaggerated example of the differences involved; but, nevertheless, one should endeavor to be more precise in one's statements of the food value of the vegetables in our diet.

## SUMMARY

*Carbohydrates.* A study of the activity of taka-diastase showed that an active maltase capable of converting maltose to glucose was present. This conversion can be accomplished if the concentration of the maltose in the substrate is not more than 0.25 gm. in 100 cc. of solution. Use of this fact was made in devising a method for determining the available carbohydrate of a food.

*Crude fiber.* A comparison of the enzymatic procedure for determining the crude fiber of spinach with the so-called official method showed that the former gives results which are more than three times as large as those obtained by the present 'official method.'

*Fats.* A method for estimating the true fat content of the ether-soluble fraction of a food material is presented. The application of this method to spinach showed that more than 55% of the material obtained by ether extraction and sometimes called 'fat' is indigestible.

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# THE EFFECT OF CYSTINE AND CASEIN SUPPLEMENTS UPON THE NUTRITIVE VALUE OF THE PROTEIN OF RAW AND HEATED SOY BEANS<sup>1</sup>

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Several investigators have analyzed the soy bean to determine the completeness of its protein. Osborne and Clapp ('07), Jones and Waterman ('21), Hamilton, Uyei, Baker and Grindley ('23), and Nollam ('15) showed that the protein of the soy bean contains in quantity all of the essential amino acids with the possible exception of cystine. Mitchell and Smuts ('32) and Shrewsbury and Bratzler ('33) claimed a quantitative deficiency of the amino acid, cystine, in the protein of the raw soy bean on the basis of biological experiments. However, Csonka and Jones ('34) reporting an analysis of a 10% sodium chloride extract of defatted soy bean meal—which represented 85 to 90% of the total nitrogen of the meal—obtained values for cystine which ranged from 0.287 to 0.491%. These values led them to question the existence of an actual deficiency of cystine in soy bean protein. They pointed out that different varieties of soy beans differed materially in their protein content. It was suggested that for practical feeding purposes it might be desirable to discriminate between different varieties in order to provide a satisfactory protein content of the ration.

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Hayward, Steenbock and Bohstedt ('36) found by metabolism trials that the protein of the soy bean was made more efficient by exposure to heat. With this exposure to heat there resulted only a slight increase in digestibility. Their results suggested that the application of heat to the raw soy bean caused an increase in the availability of certain fractions of the protein molecule. It seemed important that these findings should be submitted to more detailed analysis. In line with this we have now sought to determine if cystine or its equivalent represents that part of the protein fraction which is made more available by a process of heating. We have centered our attention primarily upon the effect of the process of heating which is used commercially in the extraction of the soy bean oil. In a supplementary way we have also carried out some experiments on the effect of casein when fed at different levels in supplementing the proteins of the raw soy bean. The primary objective of these experiments was to demonstrate if the beneficial effect of heat was exerted on the protein fraction only or whether the digestibility and availability of other constituents of soy beans were likewise improved.

#### EXPERIMENTAL

In our first series of experiments we fed a supplement of cystine with both raw and heated soy beans. The soy beans were of the Illini and Herman varieties. These were used because Csonka and Jones ('34) have reported that the Illini and Herman varieties differed considerably in their cystine content. We did not analyze our samples for cystine, but analyses for sulfur revealed that the Illini beans contained 0.344% sulfur and the Herman beans 0.382%. When extracted soy beans were fed, soy bean oil was added to restore the original fat content. The soy bean oil meal fed was the high temperature expeller meal prepared from Illini beans of the same sample as those fed in the control rations. The method of preparation of this expeller meal has already been published (Hayward, Steenbock, Bohstedt, '36).

In our second series of experiments casein was incorporated into the soy bean diet in the form of crude casein. Cystine was also added to check the completeness of the diet containing the raw soy beans and the intermediate level of casein.

All experimental diets were compounded so as to contain approximately 18% of protein. Actual analyses revealed a range in protein content from 17.6 to 18.3%. The basal ration in which the soy bean preparations were fed was composed of 45 parts cooked starch, 4 parts of salts number 40 (Steenbock and Nelson, '23), and 2 parts of cod liver oil. The amount of starch incorporated in the respective rations was adjusted inversely with the amount of soy bean products or other supplements required by analyses to supply the desired level of protein. l-Cystine was added as desired at a level of 0.3% of the ration. When yeast was added it was included at a 6% level.

Twenty-six lots of six male rats weighing from 50 to 60 gm. were used in the experiments. Each rat was quartered and fed separately. The food consumption of all rats was equalized from week to week. This was effected by reducing the intake of all rats to the intake of the rat which consumed the least, providing that this rat was not obviously abnormal (Steenbock, Black and Thomas, '29, '30). Each experiment was continued for 56 days. The results of the experiments are grouped together in table 1. They are expressed in terms of gain in body weight per gram of protein eaten (Osborne, Mendel and Ferry, '19). The average daily food consumption of all rats was 4.84 gm., which represented the amount consumed by the rats in lot 52 which received the raw soy bean diet.

It is to be noted that the ground raw soy beans of both the Illini and Herman varieties showed a low nutritive value as indicated by the grams of growth per gram of protein consumed. The addition of cystine or autoclaving the beans at 15 pounds pressure for 1 hour practically doubled the nutritive value of both varieties. Similarly, high temperature heating, as used in the expeller process of oil extraction, pro-

TABLE 1

Series 1: The effect of supplements of cystine, casein and yeast upon the nutritive value of the protein of raw and heated soy beans

LOT	DIET			GAIN, BODY WEIGHT		GROWTH PER GRAM OF PROTEIN EATEN
	Soy beans		Special supplements	Range	Average	
	Kind <sub>a</sub>	Process				
52	Ground beans A			Yeast <sup>1</sup>	gm. 8-26	gm. 0.38
54	Ground beans A			Cystine	20-37	18.6
56	Ground beans A			Cystine	28-64	28.2
57	Ground beans A			Cystine	31-44	41.0
58	Oil meal	Expeller	150° C.	Yeast <sup>1</sup>	28-50	36.8
59	Oil meal	Expeller	150° C.	Yeast <sup>1</sup>	27-45	40.7
62	Oil meal	Expeller	150° C.	Cystine	40-53	38.7
63	Oil meal	Expeller	150° C.	Cystine	38-46	46.5
77	Ground beans A	Autoclaved	15 pounds 121° C. 1 hour	Yeast <sup>1</sup>	40-58	40.7
79	Ground beans A	Autoclaved	15 pounds 121° C. 1 hour	Cystine	40-58	50.4
70	Ground beans H				43-59	49.7
71	Ground beans H			Yeast <sup>1</sup>	19-30	26.2
72	Ground beans H			Cystine	28-35	30.2
73	Ground beans H			Cystine	37-51	44.5
76	Ground beans H			Cystine	37-44	40.8
78	Ground beans H	Autoclaved	15 pounds 121° C. 1 hour	Cystine	46-62	55.0
53	Ground beans A	Autoclaved	15 pounds 121° C. 1 hour	Cystine	41-59	50.5
55	Ground beans A			Casein 2½%	29-36	31.1
64	Ground beans A			Casein 2½%	27-39	32.7
65	Ground beans A			Casein 5%	24-44	36.8
66	Ground beans A			Casein 5%	30-42	37.2
67	Ground beans A			Casein 10%	40-51	46.2
68	Ground beans A			Casein 10%	42-53	47.8
69	Ground beans A			Casein 5%	52-56	52.2
74	Oil meal	Expeller	150° C.	Cystine	38.51	44.0
75	Oil meal	Expeller	150° C.	Cystine	35-53	45.3
				Yeast <sup>1</sup>	37-51	43.8

<sup>a</sup> Soy bean oil added in minor amounts.

<sup>b</sup> The Illini variety of soy beans are designated with the letter 'A' and the Herman variety with the letter 'H.' The expeller soy bean oil meal was prepared from the Illini variety.

duced a decided increase in nutritive value. With the heated soy bean products, the addition of cystine did not produce unequivocal results; the nutritive value was apparently slightly improved with the expeller meal but was left unchanged in one of the autoclaved meals and somewhat reduced in the other. Evidently, the variations can be considered as due to experimental error. The addition of yeast to the raw soy bean diets caused a slight improvement in nutritive value, but with the diets containing the heated soy beans or the raw soy beans supplemented with cystine, yeast not only failed to improve the nutritive value of the protein but actually decreased it slightly. This was probably caused by the reduction in the amount of soy bean protein in the ration, which was made to keep the protein level constant. Apparently, yeast protein was not on a parity in nutritive value with the protein of the heated soy beans or the raw soy beans plus cystine. The fact that the addition of yeast to our heated soy bean rations did not produce any response in growth confirmed our surmise that our rations were well supplied with the vitamins B.

The addition of casein to the raw soy bean diets caused an improvement in nutritive value. This increase was in proportion to the level of casein fed. When, in addition to 5 parts of casein, 0.3% of l-cystine was added, the nutritive value of the ration was again materially increased. In amount this was about the same as that produced with the raw soy beans upon the addition of 10 parts of casein. The incorporation of 2½% of casein in the heated soy bean diet failed to cause any material change.

#### DISCUSSION

The fact that cystine acts as an efficient supplement to the protein of the raw soy bean as also reported by Mitchell and Smuts ('32) and Shrewsbury and Bratzler ('33) might lead to the conclusion that soy bean protein is generally deficient in cystine. However, Csonka and Jones ('34) reported that soy bean oil meal contained from 0.287 to 0.491% of cystine when prepared, respectively, from the Illini and Herman

varieties. These findings of Csonka and Jones and our sulfur values for the samples of soy bean of the Illini and Herman varieties used in our experiments seemed to us good reasons for suspecting that while cystine might be a limiting amino acid for the ray sow bean, this was not due to an actual deficiency but rather to a lack of availability of cystine or its equivalent. Of course, it is not excluded that cultural conditions have an effect upon the cystine content of soy beans and that other varieties than those used by us might even show greater departure from the values given. Conclusions in regard to this are not warranted until a general survey has been made. As our data are limited to the results obtained with samples of only two varieties, we can merely state that in some instances cystine or its biological equivalent is present in raw soy beans, at least in part in an unavailable form. Heating the soy bean apparently serves to correct or alleviate this deficiency.

The fact that the addition of yeast to the raw soy bean diets supplemented the proteins of soy beans to some extent is not surprising in view of the cystine content reported for yeast. Prunty ('33) analyzed various samples of yeast dried at 37°C. and found them to contain from 0.51 to 0.92%. Swift, Kahlenberg, Voris and Forbes ('34) found that yeast added at the rate of 1 gm. per rat per week to a diet purposely constructed to be deficient in cystine caused the rats to gain weight as rapidly as their mates which received a diet known to be satisfactory in cystine content. This additional gram of yeast per week was estimated to raise the cystine intake of the basal diet by about 12%. They attributed the increased growth to the cystine content of the yeast. We did not analyze our sample of yeast for cystine, but found that it contained 0.396% of sulfur.

Jackson and Block ('32) have reported that the amino acid methionine, like cystine, is capable of producing good growth in rats when added to a basal diet poor in cystine. Baernstein ('32) reported a value of 1.84% for methionine in glycinin—the isolated protein of the soy bean. He also reported that casein contained from 3.25 to 3.53% of methionine.

When we consider the cystine content of our soy bean diets in terms of the cystine values obtained by Csonka and Jones ('34) and the methionine value of Baernstein ('32), we obtain an appreciable value, viz., 0.25 to 0.35%. These differences in cystine equivalent values are offered as an explanation for the increased nutritive value obtained for the soy beans H, lot 76, over that for the soy beans A, lot 77, when both beans had received the same heat treatment, autoclaved 1 hour at 15 pounds pressure. These cystine equivalent values would be sufficient to explain why the diets containing the heated soy beans A and H failed to respond markedly to additions of cystine, if we assume that heating made this cystine equivalent fraction available for absorption and ultimate use in the animal's body. To support this supposition, other investigators (Rose, '31, '32; Jackson and Block, '32; and Swift, Kahlenberg, Voris and Forbes, '34) have found that the incorporation of 0.24 to 0.3% of l-cystine in a cystine deficient diet was sufficient to produce normal growth providing the diet was complete in other essentials.

It is known that casein ordinarily contains a variable but appreciable amount of cystine (Folin and Looney, '22; Jones and Gersdorf, '34) and methionine (Baernstein, '32). The results with different levels of casein then are not surprising in view of the cystine equivalent values for this protein and the fact that the protein of the raw soy bean can be supplemented by even small additions of available cystine as was shown in table 1.

The results of these experiments suggest that cystine or its equivalent may exist in the raw soy bean in a form which is at least partially unavailable to the rat. The application of heat, as for example, autoclaving the beans for 1 hour at 15 pounds pressure, or the use of the expeller method of oil extraction which involves heating the beans for 2½ minutes at a temperature of 140 to 150°C., caused the limiting factor to become available. Whether these differences lie in a failure of absorption or in a lack of availability after absorption remains to be demonstrated.

## SUMMARY

In experiments with rats, raw soy beans of both the Illini and Herman varieties were found to contain an inefficient protein as measured by grams of growth per gram of protein eaten.

The additions of 0.3% l-cystine or the application of heat, such as autoclaving for 1 hour at 15 pounds pressure or the use of the expeller method of oil extraction, which entails heating the beans at a temperature of 140 to 150°C. for 2½ minutes, practically doubled the nutritive value of the protein. The addition of 0.3% of l-cystine to the heated soy bean diet failed to reveal an improvement which could be considered outside of possible experimental error.

Casein supplemented the protein of the raw soy bean and caused an increase in the nutritive value of the protein proportional to the amount added. The addition of 0.3% l-cystine resulted in an increase in nutritive value similar to that obtained when the high level of casein was added.

The fact that cystine supplemented the protein of the raw soy bean suggests that cystine or its equivalent may exist in the raw protein of the soy bean in a form which is not available to the animal. Since heating of the soy bean gave its protein a nutritive value practically equal to the nutritive value of the protein of the raw soy bean when supplemented with cystine, it appears that heating the soy bean caused the cystine fraction of the protein to become available.

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## LOSSES OF VITAMIN C DURING THE COOKING OF PEAS<sup>1</sup>

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### FOUR FIGURES

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Certain well-known investigators have reported that open-kettle cooking of vegetables is very destructive to vitamin C. Eddy, Kohman and Carlsson ('26) found 2 gm. daily of ungraded raw market peas, and 5 gm. but not 3 gm. of these ungraded peas cooked, protective against scurvy in guinea pigs. This gives a loss of approximately 75% of vitamin C during cooking. Bessey and King ('33) report, by dye titration, 0.16 mg. of vitamin C per milligram of fresh peas and 0.06 mg. per gram of cooked peas. McHenry and Graham ('35) found 0.14 mg. per gram of raw peas and 0.08 mg. per gram of cooked peas or a cooking loss of 43%. Fellers ('35) lists peas in a group of vegetables losing 40 to 80% of their vitamin C "during a short cooking period." He reports 3.6 gm. of cooked peas as the protective level. Recalculated, on a basis assuming 0.5 mg. of ascorbic acid per day are required to protect a guinea pig from scurvy, this is 0.14 mg. of vitamin C per gram of cooked peas. He gives no data on the raw pea content.

In the studies on peas published previously, no consideration has been given to the cooking water, perhaps partly

<sup>1</sup>Approved by the director of the New York State Agricultural Experiment Station for publication as Journal Paper no. 128, March 11, 1936.

because it is difficult to get animals to eat the large amount of cooking water produced. The new chemical method offers a very simple means of determining the amount of ascorbic acid that goes into the water. Our purpose was to determine not only the ascorbic acid retained in the peas, but also that retained in the cooking water. From the standpoint of human nutrition, the results of animal feeding experiments do not give as exact information as do the chemical determinations, inasmuch as human beings commonly eat food before it is cooled.

In this study it was possible to control the variety, maturity, and freshness of the peas, as well as the method of cooking. The main object was to measure the relative amounts of vitamin C in the peas and in the cooking water at the 'done' stage and at various intermediate stages, as well as losses during the standing of cooked peas. Every effort was made to control all of the factors in such a way as to cause minimum loss of vitamin C with the exception that the aim in cooking was to make the cooked vegetable look attractive and taste well, with the loss of nutrients a secondary consideration.

#### EXPERIMENTAL

1. *Losses of vitamin C during the cooking of peas.* Two varieties of peas, Thomas Laxton and Alderman, were grown on upland soil (Ontario clay loam at Geneva, New York). They were hand-picked when they reached the succulent stage, hand-shelled, cooked, samples extracted, and titrated in as short a time as possible.

The method of cooking was essentially that of Halliday and Noble ('33), with the exception that 525 gm. (six servings) of peas instead of 350 gm. were used with the 700 cc. water and  $\frac{3}{4}$  teaspoonful salt. A 3-quart enamel pan with an inside diameter of 7 inches was used. The peas were dropped into the rapidly boiling water and the time counted from the time the water came back to the boil (this took 2 minutes). The tap water (pH approximately 7.5) was boiled several minutes before the peas were added.

An arbitrary stage of 'doneness' for each variety was determined in advance by several judges, using the common household methods of testing with a fork and 'biting.' The boiling time for the Thomas Laxton and the Alderman varieties was 14 and 13 minutes respectively. Samples of the peas and of the cooking water were removed at intervals from the beginning of cooking to the 'overdone' stage. Samples were also taken at the 'done' stage from normal cookings in which there had been no interference from previous sampling.

The method of extraction, of standardization of the dye, and titration were essentially those of Bessey and King ('33). Trichloroacetic acid<sup>2</sup> was found to give a clearer extract of peas than acetic acid. Blanks were run with each dye used.

The samples of peas containing approximately 20 gm. were placed immediately into weighing bottles containing 25 cc. of 8% trichloroacetic acid. The weighing bottles containing the acid had been previously placed in an ice-salt mixture so that when the hot peas were added to the chilled acid, the whole was brought to room temperature in a very short time.

Immediately after removal of each sample for vitamin C determinations, samples were removed for moisture determinations so that the vitamin content was calculated on both the wet and the dry weight.

The cooking water in 25 cc. amounts was pipetted into 25 cc. chilled trichloroacetic acid in 50 cc. volumetric flasks.

There was little change of acidity, which might affect vitamin C stability, during the cooking. The total range of pH of the raw and cooked peas was from 6.30 to 6.88, indicating the presence of considerable buffer material in peas. The pH of the tap water used in cooking and of the cooking water at the 'done' stage was approximately 7.5 and 6.5 respectively.

For purposes of comparison, biological assays were made on the raw and the 'done' peas. The whole lot of one variety for animal feeding was picked and cooked on the same day.

<sup>2</sup> Mack, Tressler and Dearborn ('36) have shown that 8% trichloroacetic acid prevents enzymic oxidation of ascorbic acid in fresh peas so that regeneration with  $H_2S$  is not necessary.

The 'done' peas were drained, cooled approximately 1½ minutes in shallow pans surrounded by ice and salt and then packed, sealed, frozen and kept in a container with dry ice.

The biological assay of the four samples of peas was based essentially upon the curative feeding test, in comparison with the response of animals receiving known amounts of the pure vitamin. The method was the same as that reported by Tressler, Mack and King ('36). Guinea pigs weighing about

TABLE 1  
*Vitamin C assay of cooked and raw peas*

TEST FOOD	WEIGHT OF PEAS FED	VITAMIN LEVEL	NUMBER OF ANIMALS	AVERAGE WEIGHT AT BEGINNING OF TEST	AVERAGE CHANGE IN WEIGHT DURING TEST	SCURVY SCORE
	<i>gm.</i>	<i>mg. per day<sup>1</sup></i>		<i>gm.</i>	<i>gm.</i>	
Thomas Laxton peas, raw	2.17	0.5	6	323	+ 34	3
Thomas Laxton peas, cooked	9.10	1.0	3	316	+ 55	0
Alderman peas, raw	2.08	0.5	5	323	+ 57	2
Alderman peas, cooked	3.33	0.5	5	322	+ 40	3
Standard solution <sup>2</sup>		1.0	6	310	+ 67	0
Standard solution		0.5	7	311	+ 41	2
Basal diet only		0.0	6	329	-113	19

<sup>1</sup> Quantity fed based upon values obtained from preliminary and concurrent indophenol titrations.

<sup>2</sup> The standard solution contained 1.0 and 0.5 mg. of ascorbic acid.

300 gm. at the time of purchase were fed a Sherman basal diet (Sherman, La Mer and Campbell, '22) (rolled oats, bran, butterfat, heated skimmilk powder, and salt), supplemented with cod liver oil and 4% yeast. A liberal allowance of crisp carrots or spinach was given during a preliminary period of 10 days, to eliminate any animals that appeared subnormal in growth rate or health. They were then kept on the basal diet without a vitamin C supplement for 11 days to deplete their tissue reserves, as indicated by a lessened or negative growth rate. The test peas were then given to matched groups in

TABLE 2  
*Vitamin C losses from peas and gain in the cooking water*

LENGTH OF COOKING PERIOD IN MINUTES <sup>1</sup>	THOMAS LAXTON VARIETY			ALDERMAN VARIETY		
	Ascorbic acid			Ascorbic acid		
	Milligram per gram drained peas		Milligram per cubic centi- meter cooking water	Milligram per gram drained peas		Milligram per cubic centi- meter cooking water
	Wet weight	Dry weight		Wet weight	Dry weight	
0 (raw)	0.23	0.99	0.00	0.24	0.95	0.00
1	0.16	0.74	0.04	0.21	0.83	0.02
3	0.11	0.50	0.05	0.19	0.74	0.03
4	0.11	0.50	0.06	0.18	0.74	0.05
6	0.11	0.48	0.07	0.17	0.69	0.06
8	0.10	0.44	0.08	0.15	0.59	0.07
10	0.10	0.43	0.08	0.15	0.60	0.08
12	0.10	0.41	0.09	0.14	0.62	0.08
14	0.09	0.40	0.11	0.13	0.53	0.10
16 <sup>2</sup>	0.08	0.38	0.11	0.12	0.52	0.12
18	...	...	0.13	...	...	0.14

<sup>1</sup>In all cases approximately 2 minutes were required to bring the water back to boiling after the peas were put into it, hence the peas cooked 1 minute had not been boiled, while those cooked 3 minutes had been boiled for 1 minute.

<sup>2</sup>Thomas Laxton peas were done in 16 minutes, the Alderman in 15 minutes.

TABLE 3  
*Vitamin C losses from cooked peas and gain in the cooking water. Samples not taken until peas were done*

LENGTH OF COOKING PERIOD IN MINUTES <sup>1</sup>	CONDITION OF PEAS	THOMAS LAXTON VARIETY				ALDERMAN VARIETY			
		Ascorbic acid			Weight of cook- ing water	Ascorbic acid			Weight of cook- ing water
		Milligram per gram drained peas		Milligram per cubic centimeter cooking water		Milligram per gram drained peas		Milligram per cubic centimeter cooking water	
		Wet weight	Dry weight			Wet weight	Dry weight		
0	Raw	0.23	1.01	0	gm. 700	0.25	0.96	0	gm. 700
15	Just done	...	...	...	...	0.15	0.57	0.12	440
16	Just done	0.11	0.44	0.13	430	...	...	...	
17	Overdone	...	...	...		0.14	0.54	0.14	
18	Overdone	0.11	0.52	0.14		...	...	...	
19	Overdone	...	...	...		0.13	0.51	0.16	
20	Overdone	0.12	0.53	0.18		...	...	...	
22	Overdone	0.12	0.54	...		0.14	0.52	0.19	
24	Overdone	...	...	0.26		...	...	...	
25	Overdone	...	...	...		0.13	0.54	0.20	
28	Overdone	...	...	0.39		...	...	...	

<sup>1</sup>In all cases approximately 2 minutes were required to bring the water back to boiling after the peas were put into it.

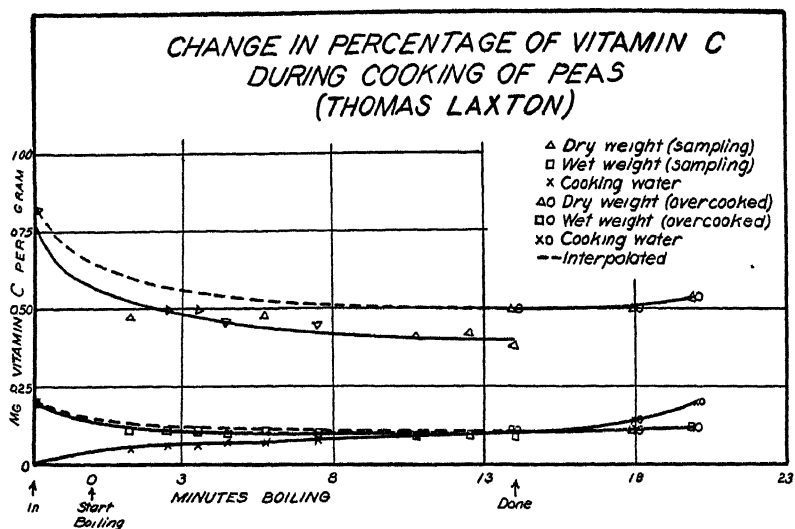


Figure 1

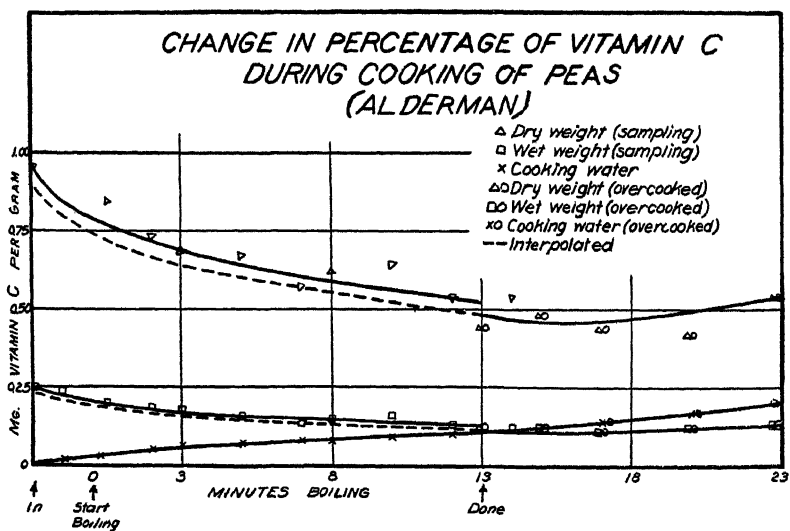
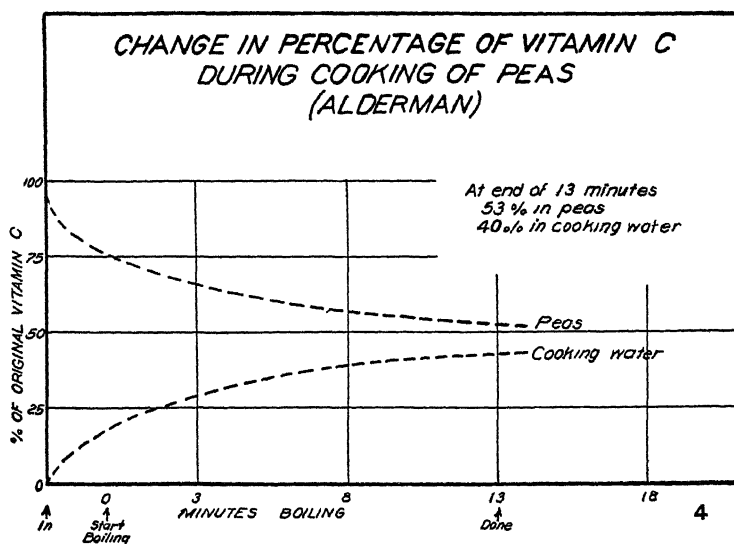
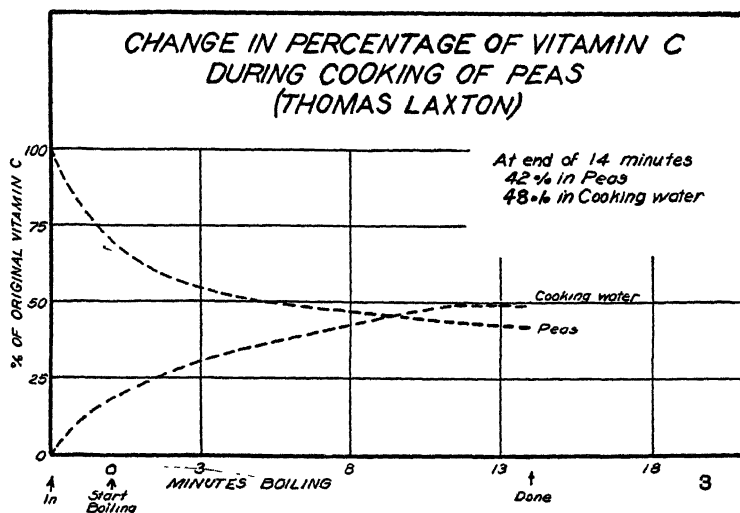


Figure 2





Figs. 3 and 4 These curves were drawn from values obtained by allowing for the vitamin C contained in the samples used for analysis. The amount of cooking water was estimated.

such quantity that, based upon preliminary and concurrent indophenol titrations, each animal received 0.5 or 1.0 mg. of vitamin per day during a period of 14 days. For comparison, other groups received 0.5 and 1.0 mg. of pure vitamin per day, fed in a standard solution from graduated pipettes. Negative controls continued to receive the basal diet only.

The results of the assay, given in table 1, show close agreement in growth response and scurvy scores for the animals receiving test peas, in comparison with those receiving standard vitamin solution. A few animals in each group failed to eat their test food promptly during the first 3 days of the assay period and were therefore discarded.

There was no significant loss in the titration value of the peas during the specified period of storage for the assay. During this period they were held in insulated fiber shipping cases with liberal quantities of dry ice.

2. *Losses of vitamin C during the standing of cooked peas.* The peas used in this part of the experiment were of the Telephone variety and were purchased on the open market the latter part of July. They were cooked in the same manner as the other varieties, but it was found necessary to boil them 19 minutes. To find the loss of vitamin C during standing, the cooked peas, in one serving amounts ( $\frac{1}{2}$  cup), were placed on dishes. The vegetables were left thus exposed to the air at room temperature and no doubt cooled very quickly. Representative samples were removed at 5-, 15- and 70-minute intervals. The 5- and 15-minute periods represent the interval between the time the vegetable is removed from the stove and the time it may be eaten. The 70-minute period represents the time the cooked vegetable may be held for animal feeding.

#### DISCUSSION

The results of titrations calculated on the wet and dry basis of the peas and the results of titrations of the cooking water are presented in tables 2 and 3. The data in tables 2 and 3 are represented graphically by figures 1 and 2. The curves so constructed show the vitamin C content of the raw

and cooked peas, the solution into the cooking water, and the destruction of vitamin C from the peas during the cooking and overcooking. They also show the total loss of vitamin C from the vegetable and the total loss to the cooking water at the 'done' and the 'overdone' stages, the peas being cooked with no interference from sampling. Figures 3 and 4 show the percentage of vitamin C retained in the cooking water and in the peas at the 'done' stage.

The vitamin C content of the raw peas, 0.23 and 0.24 mg. per gram for the Thomas Laxton and the Alderman, respectively, was about the same as the 0.25 mg. per gram (recalculated from biological tests) reported by Eddy, Kohman and Carlsson ('26), but was higher than that reported by Bessey and King ('33) 0.16 mg. per gram (by dye titration). In both of these cases, the product was of uncertain variety and was purchased as a typical market vegetable. The higher content is partly explained by the fact that the peas were picked at the succulent stage and that a minimum length of time elapsed before they were tested. There is also the possibility that the one group of workers may have used varieties of peas lower in vitamin C. In a test of the vitamin C content of eighteen varieties of peas, Mack, Tressler and King ('36) found the Thomas Laxton midway in the group and the Alderman in the lower third. During cooking, the Alderman variety retained vitamin C in the peas to a slightly greater extent than did the Thomas Laxton.

In every case the greatest loss of vitamin C from the vegetable was during the first 2 minutes of cooking (the time it took the water to return to the boiling point). A possible explanation of the large initial loss is that the enzyme which catalyzes the oxidation of vitamin C was inactivated during this short period. Another factor may be that as soon as the oxygen was driven out, there was an atmosphere of steam above the water, and subsequent oxidation was decreased.

The rate of loss of vitamin C after the water came back to the boil was very low and decreased almost to zero.

The large amount of vitamin C dissolved in the cooking water is worthy of notice. This amount increased almost linearly with time. Vinokurov and co-workers ('35) and Halliday and Noble ('36) also found large amounts of vitamin C lost from vegetables to the cooking water. The vitamin C content of the cooking water from peas (0.10 to 0.12 mg. per cubic centimeter) compares favorably with that reported in the literature for tomato juice (0.14 to 0.27 mg. per cubic centimeter).

The total destruction of vitamin C in the Thomas Laxton at the 'done' stage was 10%, 42% being retained in the peas and 48% in the cooking water. The total destruction in the Alderman was 7%, 53% being retained in the peas and 40% in the cooking water.

The slight actual destruction of vitamin C may be partially explained by the low pH of the peas, which remained less than 7 throughout the cooking period. Barron, De Meio and Klemperer ('36) report "ascorbic acid is not autoxidizable in acid and neutral solutions up to pH 7.0."

Upon being overcooked, the peas apparently increased slightly in vitamin C, both on the wet and dry percentage basis. At this point, the peas and the cooking water were both reduced to a small amount, so that the rate of evaporation from the cooking water was very rapid. Another possible factor may have been introduced by the formation of small quantities of non-vitamin dye-reducing substances.

During the standing of the cooked peas, the destruction was very slight, but was greatest during the first 5 minutes, amounting to about 9% on the wet weight basis; at the end of 70 minutes, the destruction had increased to 14% of the vitamin C content of the 'done' peas. It is probable that this small destruction was caused by atmospheric oxygen and occurred largely in the peas on the surface. Consequently, after the preliminary destruction, very little further decomposition took place.

## SUMMARY

1. The work shows clearly the value of the cooking water of peas from a nutritive standpoint, since about one-half of the vitamin C passes into it.
2. The greatest rate of loss of vitamin C from the peas occurred during the first 2 minutes of cooking.
3. The question of a few minutes of overcooking or undercooking of peas is not an important factor.
4. The loss of vitamin C during standing of the drained cooked vegetable at room temperature is relatively small and seems to occur during the first few minutes.

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## THE RELATION OF VITAMIN G<sup>1</sup> TO THE HATCHABILITY OF HENS' EGGS

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In previous reports (Bethke and Kennard, '28, '30) we pointed out that the ration for the breeding flock must contain the factor or factors carried by milk, fresh grass, or alfalfa leaf meal, in addition to vitamin D, for the production of eggs of high hatchability. Other investigators have also reported that diet is a factor in embryonic development. Byerly, Titus and Ellis ('33 a) found that diets which contained only feed stuffs of vegetable origin yielded eggs in which a relatively large percentage of the embryos died during the second week of incubation, associated with a high incidence of chondrodystrophy. The same authors (Byerly, Titus and Ellis, '33 b) later reported that corn, wheat, ground soy beans, soy bean meal, cottonseed meal, flame-dried menhaden meal and some lots of meat meal are deficient in some substance necessary for the production of eggs capable of supporting embryonic life during the second week of incubation. On the contrary, vacuum dried North Atlantic fish meal, steam-dried crab meal, dried buttermilk, and a combination of dried buttermilk, North Atlantic fish meal and meat meal, and free range were found by these investigators to enable the production of eggs capable of supporting embryonic life through the second week of incubation in normal fashion. The authors state:

<sup>1</sup> Vitamin G(B<sub>2</sub>) as used in this paper has reference to the complex and not to any one of the several factors which make up its complexity.

<sup>2</sup> Published with the permission of the director of the Ohio Agricultural Experiment Station.

Evidence as to the nature of the deficiency is somewhat conflicting. The proteins in the deficient concentrates may well have been qualitatively deficient. It is possible that vitamin G, or some one of the less known water soluble vitamins, is concerned, although the authors have thus far been unable to obtain experimental evidence for such a view.

McFarlane, Fulmer and Jukes ('30), in studies of embryonic mortality in chicks found that the source of protein in the ration of the parent stock exerted a marked influence on mortality, which was not associated with the total nitrogen, amino acid, ash, or iron content of the egg. Calvery and Titus ('34) were also unable to find differences in the amino acid makeup of eggs from birds kept on different rations. On the contrary, Titus, Byerly and Ellis ('33) reported data which they interpreted as indicating differences in the crude protein contents of eggs due to the ration fed the hens producing them.

Investigators at the Ontario Agricultural College (Anonymous contribution, '35) found that the feeding of buttermilk, alfalfa leaf meals, and liver extract decreased early embryonic mortality and increased hatchability of the eggs. Halpin, Holmes and Hart ('33) reported that the addition of skimmilk, chopped alfalfa hay, or autoclaved yeast to the ration of laying hens increased the hatchability of the eggs produced by supplying vitamin G. Heiman ('35) also attributed the increased hatchability resulting from the addition of dried skimmilk or dried whey to a cereal-casein ration to the vitamin G content of the milk products added.

The experiments reported in the present paper were undertaken to confirm and extend our earlier studies on the effect of the ration on the ability of eggs produced to sustain good embryonic development. The data presented were obtained during the 4-year period, 1930 to 1934.

#### EXPERIMENTAL

In all trials White Leghorn yearling hens of known pullet year egg production were used. These were mated with cockerels of the same breed several weeks before the eggs



were saved for incubation purposes. The males in any particular experimental series were rotated three times each week between the different pens, in order to overcome individual differences. All groups of birds were kept indoors in small pens, provided either with straw or wood shavings as litter. The trials were started either the latter part of November or the fore part of December and continued until May or June. Ten females and one male were placed in each pen. The incubation studies were started from 4 to 6 weeks after the hens had been put on their experimental rations. All eggs were set in the same electrically operated incubator, at weekly intervals for a 3- to 5-month period.

The basal rations used in the different trials were of the same general composition except for the source of supplemental protein. They consisted of: Ground wheat, 15.0; wheat bran, 10.0; steamed bone meal, 2.0; fine oyster shells, 4.0; salt, 0.5; cod liver oil, 1.0 to 2.0; either meat scraps, 10.0; menhaden fish meal, 9.0; or casein, 7.5, and yellow corn to make 100 parts. The rations were mixed fresh approximately every 2 weeks, in order to avoid vitamin deterioration. Oyster shells were also kept before the birds in open feeders.

In the first trial a basal ration containing meat scraps was fed. This was supplemented with alfalfa leaf meal, an acidulated water extract of alfalfa leaf meal, dried skim-milk, a milk serum concentrate, and autoclaved yeast. The alfalfa leaf meal replaced an equivalent quantity of wheat bran in the basal mixture. The protein content of all rations was equalized by decreasing proportionately the amount of meat scraps. The yeast was prepared by autoclaving a pure dried bakers' yeast for 6 hours at 15 pounds pressure. The alfalfa extract was made by treating the alfalfa leaf meal with four separate portions of slightly acidulated water and concentrating to a suitable volume before electric fans at room temperature. The milk serum concentrate was obtained from the Dry Milk Company, of Bainbridge, New York. According to the manufacturers, it represents a concentration of the milk serum after the removal of the fat, casein, lacto-albumin, and greater part of the milk sugar and part of the

calcium phosphate, to the consistency of molasses. This concentrate has been shown to be a good source of the vitamin-B complex by Supplee, Dow and Flanigan ('28) and to have growth promoting and leg paralysis preventing properties for chicks by Norris and associates ('30) and Bethke et al. ('31). We have also found it to be a good source of vitamin G(B<sub>2</sub>), as determined on rats.

The results of the incubation trials presented in table 1 clearly show that alfalfa leaf meal, dried skimmilk, and autoclaved yeast carry some factor or factors essential for good hatchability, or the proper development of the embryo. In

TABLE 1

*Effect of alfalfa leaf meal, water extract of alfalfa, milk and autoclaved yeast on hatchability*

PEN	ADDITIONS TO BASAL RATION	EGGS SET	FERTILE	CHICKS HATCHED	FERTILE EGGS HATCHED
		no.	%	no.	%
1	None	357	90.2	97	30.1
2	5.0% alfalfa leaf meal	459	96.7	298	67.1
3	Water extract of 5.0% alfalfa leaf meal	356	95.5	216	63.5
5	7.5% dried skimmilk	579	95.3	395	71.5
6	2.5% milk serum concentrate	306	93.1	180	63.2
8	1.0% autoclaved yeast	308	96.7	149	50.0
9	3.0% autoclaved yeast	370	89.7	235	70.8

this respect the results with milk and alfalfa confirm our earlier observations (Bethke and Kennard, '28, '30) on this problem. The results obtained with the aqueous extract of alfalfa leaf meal and the milk serum concentrate indicated that the hatchability promoting factor or factors are water soluble and that supplemental protein was probably not concerned in the results secured. The fact that the above products, which gave an increase in hatchability, had been found to be good sources of vitamin G in our laboratory and by other investigators suggested that this factor was essential for embryonic development. Accordingly, our next step was to determine whether liver, which had been shown to be an

excellent source of vitamin G and dried whey, which represented a greater concentration of the water-soluble constituents of milk than dried skimmilk, carried the factor or factors conducive to good hatchability. There was also included one pen on wheat germ and another on wheat germ oil, to determine the effect of additional vitamin E. Although we had experienced no difficulty in obtaining good fertility in the eggs, it was thought advisable to include vitamin E, in view of the more recent experiments on the relation of limited intakes of this factor to nutrition and reproduction. The liver meal, which was prepared from beef liver, and the dried whey replaced equivalent quantities of the meat scraps protein, and the wheat germ an equal amount of wheat bran in

TABLE 2

*Effect of liver meal, wheat germ, wheat germ oil and dried whey on hatchability*

PEN	ADDITIONS TO BASAL RATION	EGGS SET	FERTILE	CHICKS HATCHED	FERTILE EGGS HATCHED
1	None	no. 297	% 87.9	no. 21	% 8.0
2	3% liver meal	708	80.9	486	84.8
4	10% wheat germ	520	91.3	145	30.5
5	2% wheat germ oil	310	90.3	6	2.1
7	5% dried whey	504	84.3	272	64.0

the basal ration. The wheat germ oil was prepared by extraction with anhydrous ethyl ether.

The data presented in table 2 show that the liver meal and dried whey exerted a marked effect on hatchability. The replacement of the wheat bran in the basal ration with pure wheat germ also increased hatchability—indicating that the germ portion of the wheat contained some of the same factor or factors present in alfalfa, yeast, liver and certain milk products. That the favorable response was not associated with vitamin E or other possible factors found in the oil of the germ is shown by the negative results in case of the eggs from pen 5.

Next we desired to determine whether some of the same products which gave an increased hatchability when added to

the meat scraps basal ration, would give similar results when some other source of protein, like menhaden fish meal and an Argentine and a domestic casein, replaced the meat product in the basal ration. The two caseins were used because in studies with chicks and rats the domestic product was found to contain significantly more vitamin G than the Argentine product. It was also desired to obtain information on the stability of the hatchability-promoting factor or factors to autoclaving in an acid and alkaline medium. Several investigators (Williams, Waterman and Gurin, '29; Guha, '31) had

TABLE 3

*Effect of alfalfa leaf meal, autoclaved yeast, liver meal and acid and alkaline autoclaved liver meal on hatchability*

PEN	MODIFICATIONS OF BASAL RATION	EGGS SET	FERTILE	CHICKS HATCHED	FERTILE EGGS HATCHED
		<i>no.</i>	<i>%</i>	<i>no.</i>	<i>%</i>
1	None	233	98.3	23	10.0
3	5% alfalfa leaf meal	285	97.5	103	37.0
5	3% untreated liver meal	393	97.7	202	52.9
6	3% acid autoclaved liver meal	434	97.0	240	57.0
7	3% alkaline autoclaved liver meal	194	95.4	8	4.3
8	Argentine casein basal ration	211	95.3	11	5.5
9	Argentine casein 5% autoclaved yeast	336	92.8	182	58.3
10	Argentine casein 3% untreated liver meal	290	97.6	168	59.4
11	Domestic casein basal ration	113	98.2	11	9.9

The basal ration of pens 1 to 7 contained menhaden fish meal, and those of pens 8 to 11 casein.

shown that vitamin G, as determined on rats, was stable in an acid but not in an alkaline, heat-treated medium. A commercial dried beef liver meal was used. The dried meal was made into a thick paste with distilled water and hydrochloric acid and sodium hydroxide added to bring the pH of the respective samples to 3.4 and 11.1 and then autoclaved for 5½ hours at 15 pounds pressure. The pH of the acid-treated meal had risen to 4.05 and the alkaline-treated product had dropped to 9.30 after autoclaving. The heat-treated meals were dried in a hot air oven at 110 to 120°C., ground, and fed as indicated in table 3. The liver meals replaced an equivalent

amount of fish meal protein in the basal ration and the alfalfa leaf meal an equal amount of wheat bran. The yeast fed to pen 9 was autoclaved for 6 hours at 15 pounds pressure at its natural pH.

The incubation data in table 3 again show that the alfalfa leaf meal, liver meal, and autoclaved yeast carried factors essential for good hatchability and that these were stable to autoclaving in an acid medium and labile in an alkaline medium. The fact that the results on the unsupplemented basal rations containing either menhaden fish meal or casein were similar to the results obtained on the unsupplemented meat scraps ration indicates that protein per se was not responsible for the increased hatchability when alfalfa leaf

TABLE 4

*Showing that the hatchability factor of dried liver was soluble in 20% alcohol*

PEN	ADDITIONS TO CASEIN BASAL RATION	EGGS SET	FERTILE	CHICKS HATCHED	FERTILE EGGS HATCHED
		no.	%	no.	%
1	None	89	92.1	0	0.0
2	3% dried liver	420	90.9	200	52.3
3	Extract of liver $\approx$ 3% dried liver	487	87.7	224	52.5
4	Liver residue $\approx$ 3% dried liver	201	83.1	0	0.0

meal, milk products, liver, and autoclaved yeast were added to the ration. This is further substantiated by the beneficial results obtained with the aqueous extract of alfalfa leaf meal and the milk serum concentrate in the first experiment. Rat and chick experiments also showed that the alkaline autoclaved liver meal was devoid of vitamin G and that the acid heat treatment of the liver product did not affect its vitamin G content.

We also found in experiments with rats and chicks (unpublished) that vitamin G could be extracted from defatted dried liver by 20% alcohol. That the factor or factors in liver essential for good embryonic development were also extracted is shown in the hatchability data in table 4. The extract used in these experiments was prepared by treating the defatted

dried pork liver with several volumes of 20% alcohol, by weight, stirring for 1 to 2 hours and allowing to stand for 12 hours, then filtering and repeating the extraction six times. The extracts were combined and reduced to an appropriate volume before fans at room temperature. The concentrated liquid was then chilled and the precipitate filtered off and added to the extracted residue. The extract and residue were fed at levels comparable to 3% of the untreated dried liver. The extract as fed supplied 90 mg. of protein (NX 6.25) per 100 gm. of ration.

Our next step was to determine whether the hatchability promoting factor of dried whey was soluble in hot 95% alcohol; since Booher ('33) had reported that such an extract contained vitamin G, as determined on rats. We also desired to determine whether vitamin B<sub>4</sub> was a factor in our hatchability results, because Keenan et al. ('33) had reported that the chick requires a factor similar to or identical with this vitamin which they found present in fresh liver and grass. The B<sub>4</sub> concentrate was prepared from fresh pork liver, according to the procedure described by Keenan et al. ('33) through the charcoal stage. The whey extract was prepared by treating the dried product with four separate portions of several volumes of 95% alcohol and refluxing under nitrogen for 2 hours. The alcohol was removed by distillation under vacuum and the residual solution allowed to stand over night in a freezing chamber, to facilitate the removal of the fat and part of the lactose. Both extracts were added to the Argentine casein basal ration. The whey extract was originally added at a level equivalent to 5% of the dried whey, but was increased to 10% 15 weeks after the experiment started. The liver preparation was fed on a 6% dried liver basis. This was replaced at the fifteenth week by 3% of dried liver autoclaved for 5 hours at 15 pounds pressure.

The results, table 5, show that vitamin G is essential for good embryonic development, since the alcoholic extract of dried whey definitely increased the hatchability of the eggs produced. The data also show that the hatchability factor

was not associated with vitamin B<sub>4</sub>. It might be assumed that the liver preparation was not fed at a sufficiently high level or that the factor was in part destroyed when mixed with the basal ration to rule out B<sub>4</sub>. This does not appear tenable, because the fresh liver dried and autoclaved, which procedure, according to Keenan et al. ('35) destroys this factor, caused a prompt increase in hatchability when it replaced the vitamin B<sub>4</sub> preparation.

TABLE 5

*Effect of liver, vitamin B<sub>4</sub>, and an alcoholic extract of dried whey on hatchability*

PEN	ADDITIONS TO CASEIN BASAL RATION	EGGS SET	FERTILE	CHICKS HATCHED	FERTILE EGGS HATCHED
		no.	%	no.	%
1	None	152	95.4	24	16.5
2	3% dried pork liver	415	94.2	327	83.6
3	B <sub>4</sub> preparation $\approx$ 6% dried pork liver	149	90.0	16	11.9
	3% autoclaved dried pork liver replaced B <sub>4</sub> preparation	83	95.2	56	70.9
4	Extract of whey $\approx$ 5% dried whey	254	91.7	72	30.9
	Extract of whey $\approx$ 10% dried whey	76	96.0	39	53.4

## DISCUSSION

The data definitely show that alfalfa leaf meal, dried skim-milk, dried whey, concentrated milk serum, autoclaved yeast, liver and wheat germ, which are fair to excellent sources of vitamin G as commonly determined on rats, exert a beneficial effect upon the embryonic life of the chick, as measured by hatchability studies. The improvement in hatchability occurred regardless of whether meat scraps, menhaden fish meal, or casein served as the main source of supplemental protein in the ration of the hen. These results, together with the observations that the factor or factors could be extracted from alfalfa leaf meal, dried liver, dried whey, and was present in milk serum practically devoid of protein indicates that the increase in hatchability was not due to protein. The finding that autoclaving of the liver meal in an alkaline medium destroyed the factor affords additional proof that the results

were not due to protein but to vitamin G. Further support that the increased hatchability was caused by vitamin G is found in the observation of Bethke and associates ('36) that eggs which showed high hatchability contained more vitamin G than those which did not hatch well.

Within recent years numerous reports have appeared in the literature which offer evidence that vitamin G, as originally considered, consists of several factors. Since the products we found to increase hatchability have been shown to be good sources of flavins, the data suggest that flavins might be concerned in the investigations reported.

#### SUMMARY

The inclusion of either alfalfa leaf meal, dried skimmilk, dried whey, autoclaved yeast, dried liver, or wheat germ in the ration of the hen caused an increase in the hatchability of the eggs produced.

It was observed that the increase in hatchability was not associated with protein and that the hatchability-promoting factor or factors were water soluble and extractable from dried liver and dried whey with cold 20% and hot 95% alcohol, respectively.

The factor or factors were destroyed in dried liver by autoclaving in an alkaline medium and were not affected at an acid reaction. The increase in hatchability was not associated with vitamin B<sub>4</sub> or vitamin E (wheat germ oil).

It is concluded that the increase in hatchability noted in the experiments reported was due to vitamin G and that this factor is necessary for the normal embryonic development of the chick.



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# THE EFFECT OF THE RATION OF THE HEN ON THE VITAMIN G<sup>1</sup> CONTENT OF EGGS WITH OBSERVATIONS ON THE DISTRIBUTION OF VITAMIN B AND G IN NORMAL EGGS

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FOUR FIGURES

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The literature contains several reports which show that the ration of the hen influences the vitamin content and the hatchability of the eggs produced. Several investigators have shown that the vitamin A and D content of eggs are correlated with the intake of these factors by the bird. Recently Barnum ('35) reported that the vitamin E content of eggs was also related to the vitamin E intake of the hen. The work on vitamin B and G(B<sub>2</sub>) has usually been directed toward the relatively potency of eggs in the several factors than toward the effect of the ration of the hen on the eggs. The earlier researches (Cooper, '21; Hoagland and Lee, '24) showed that eggs contained the antineuritic vitamin. Later work by Chick and Roscoe ('29) and Akyroyd and Roscoe ('29) revealed that egg white is devoid of vitamin B(B<sub>1</sub>) but is rich in vitamin G while egg yolk contained both factors.

More recently Ellis and associates ('33), in studies on the effect of diet on egg composition, found that the vitamin B content of the diet influenced the amount of this factor in the eggs. The results on vitamin G were not so clear cut. The authors state in part:

<sup>1</sup> Vitamin G(B<sub>2</sub>) as used in this paper has reference to the complex and not to any one of the several factors which make up its complexity.

<sup>2</sup> Published with the permission of the director of the Ohio Agricultural Experiment Station.

The results as a whole suggest that the hens' diets did not affect the vitamin G content of the eggs to the extent which has been noted for vitamin B. Lack of vitamin G in the diet apparently did not result in any pronounced drop in the vitamin content of the egg. On the other hand, the inclusion of foods rich in vitamin G in the normal diet used in these experiments tends to enhance the vitamin G content of eggs.

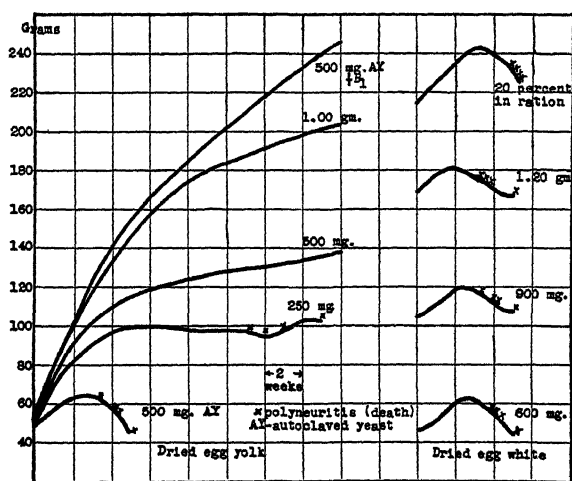
The object of the present studies was twofold: 1) to confirm the observations of Chick and Roscoe ('29) and Aykroyd and Roscoe ('29) on the distribution of vitamin B and G between egg white and egg yolk, and, 2) to determine the effect of the ration of the hen on the vitamin G content of the eggs and to ascertain whether there was a correlation between vitamin G potency and hatchability. We (Bethke, Record and Kennard, '36) had observed that the addition of products rich in vitamin G to the basal ration caused an increase in the per cent of fertile eggs that hatched and that the same products increased growth and prevented the occurrence of a leg disorder in chicks (Bethke, Record and Kennard, '31). These latter observations suggested that vitamin G was essential for the nutrition of the chick, as well as for its embryonic development, and that the high embryonic mortality on certain rations might be related to the amount of vitamin G in the egg.

#### DISTRIBUTION OF VITAMIN B AND G IN THE EGG

For the determination of the relative distribution of vitamin B and G between egg white and egg yolk we used eggs from hens on a good practical ration of corn, wheat, oats, meat scraps, milk, alfalfa meal and cod liver oil. The whites and yolks were carefully separated by hand and dried at room temperature before electric fans. Weanling rats of our own rearing were used as the experimental animals. These were confined in individual wire cages and fed a basal ration of: Extracted casein, 18; cornstarch, 64; salt mixture, 4; hydrogenated vegetable oil,<sup>3</sup> 10; agar, 2, and cod liver oil, 2. In

<sup>3</sup> Crisco.

determining vitamin B 500 mg. of autoclaved yeast was fed daily and separately to each animal, plus varying amounts of the dried egg products. For vitamin G determination, an 80% alcoholic extract of rice polish, equivalent to 1 gm. of polish, was fed daily and separately as a source of vitamin B in addition to the dried egg products. In each series there were included appropriate negative and positive control groups. Not less than four animals were included in each group.



**Fig. 1 Vitamin B in dried egg white and egg yolk.**

The results obtained on vitamin B are shown in figure 1. It is apparent that egg white is devoid of vitamin B and that the yolk contains all of this factor found in the egg—confirming the observations of Chick and Roscoe ('29) and Aykroyd and Roscoe ('29). The rats on the larger amounts of dried egg white would frequently not consume all of their supplement, so a fourth group was fed a ration in which 20% dried egg white and 10% autoclaved yeast replaced the casein and part of the cornstarch in the basal ration. The performance of this group (fig. 1) was similar to the others fed dried egg white—all animals dying from polyneuritis.

The results on vitamin G, figure 2, show that this factor is found in the egg white and egg yolk—the dried egg white containing from two to two and one-half times as much as the dried yolk. On a fresh or edible basis, the egg yolk would be somewhat more potent in vitamin G than the egg white. All of the animals in the negative control (rice polish extract), 100 mg. dried egg white, 100 and 250 mg. dried egg yolk groups developed skin lesions about the eyes and mouth and frequently these lesions extended to the head, feet and legs. No harmful effects from egg white as reported by Parsons ('31) were noted, except a thinning of the hair coat in two of the animals on the 700 mg. dried egg white level.

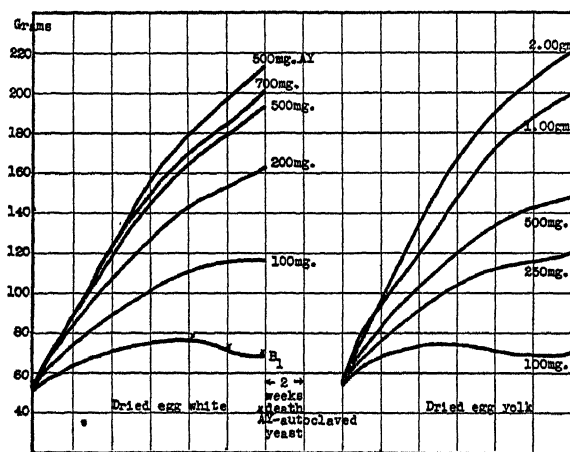


Fig. 2 Vitamin G in dried egg white and egg yolk.

#### EFFECT OF RATION ON THE VITAMIN G CONTENT OF EGGS

The preliminary studies on the effect of the ration of the hen on the vitamin G content of the eggs produced were carried out only on rats; while in the later investigations both rats and chicks were used. The eggs fed were obtained from hens which were used for hatchability studies. The hens in the different experimental groups had received the same basal ration of yellow corn, wheat, wheat bran, bone meal, oyster shells, salt, and cod liver oil except for source

of supplemental protein or vitamin G supplement for 3 or more months. Further details concerning the hen rations and management procedures are given by Bethke, Record and Kennard ('36).

#### RAT EXPERIMENTS

Rats of our own rearing, weighing from 40 to 50 gm., were confined in individual wire cages and fed a basal ration composed of: Vitamin-free casein, 18; cornstarch, 64; salt mixture, 4; hydrogenated vegetable oil,<sup>4</sup> 10; agar, 2, and cod liver oil, 2. Vitamin B was supplied in the form of an 80% alcoholic extract of rice polish. An amount equivalent to 500 mg. of rice polish was fed daily to each animal. In the preliminary trials the rats were placed directly on experiment without a depletion period; while in the later experiments the animals were kept on the basal synthetic ration, plus vitamin B for 3 weeks or longer until their weights were stationary or slightly declining over a period of 5 or more days before supplemental feeding was begun. The eggs were fed either dried or in the fresh state diluted with an equal amount of distilled water to facilitate feeding. The dried samples were prepared by thoroughly mixing the yolks and whites and drying in shallow pans at room temperature in a semi-darkened room before electric fans. In each series litter mates were kept on the basal synthetic ration plus vitamin B as negative control groups and in several instances groups receiving either autoclaved yeast or dried pork liver and vitamin B were included for positive control purposes.

The results of the preliminary trial, shown in figure 3, reveal a difference in the vitamin G content of the eggs. The rats fed the eggs produced by hens given the basal ration weighed, on the average, 20 and 48 gm. less, respectively, at the close of the experiment than their litter mates fed the eggs from hens on the same basal ration supplemented with 5% dried whey or 3% liver meal. It is also of interest to note that the growth response of the rats was correlated

<sup>4</sup> Crisco.

with the per cent of fertile eggs that hatched and the vitamin G supplement fed the hens. The eggs produced by hens on the basal ration showed the least vitamin G content and the lowest hatchability (8.0%) whereas the eggs from the liver-fed pen showed the highest vitamin G value and the highest hatchability (84.8%); and those eggs from the dried whey pen occupied an intermediate position with respect to vitamin G content and hatchability (64.0%).

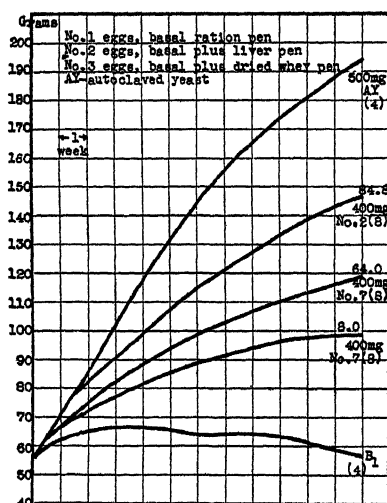


Fig. 3 The vitamin G content of eggs as affected by the addition of 3% dried liver or 5% dried whey to the ration of hens. The figures above the curves represent the per cent of fertile eggs that hatched. The figures in parentheses below the addenda indicate the number of rats in the group.

The foregoing results strongly suggested that the vitamin G content of the egg was associated with embryonic development and led us to investigate further the relation between the hatchability of eggs as affected by the ration and the vitamin G content of the eggs. The rats employed in these studies were first depleted of their vitamin G reserves, as previously indicated, before being placed on experiment. The eggs used for feeding represented random samples from different pens of hens. The eggs in series I were from hens fed either a menhaden fish meal or casein basal ration and the



same rations, respectively, supplemented with 3% of dried pork liver and 5% autoclaved yeast. The series II eggs were from birds on the same basal ration except for different types of fish meals and the basal ration containing menhaden fish meal and 5% commercial liver meal. These eggs were used because the incubation results had previously shown marked differences in the per cent of eggs that hatched. In series III the eggs were produced by hens which received a casein basal ration supplemented either with 3% dried pork liver, 3% autoclaved dried pork liver, or the 95% hot alcoholic extract of dried whey equivalent to 10% of the whey powder. The eggs in this series were fed fresh and dried to determine whether drying at room temperatures in a semi-darkened room affected vitamin G.

The results presented in graphic form in figure 4 show that the vitamin G content of the eggs was affected by the ration of the birds producing them. Without exception, those rations which contained products (dried whey, yeast, liver) which are known to be good to excellent sources of vitamin G yielded eggs that were more potent in vitamin G and that hatched well. It is also evident that there was a correlation between the hatchability of the eggs and their vitamin G content; since almost without exception the higher the per cent of hatch the greater was the vitamin G value. The results obtained on the eggs produced on the different fish meal rations (series II) also show differences in vitamin G content which are in accord with the results of the other egg studies and the hatchability results. The eggs from the menhaden fish meal-liver meal pen were not incubated; however, from results obtained on similar rations previously (series I as well as other trials), we are confident these eggs would have shown good hatchability.

#### CHICK EXPERIMENTS

The experiments with chicks were carried out to determine whether this species would show similar differences in the vitamin G content of eggs as did the experiments with rats.

The chicks used were White Leghorns hatched from eggs produced by hens fed a good practical ration. They were taken directly from the incubator and placed in brooders provided with wire grid floors and fed a basal ration of ground

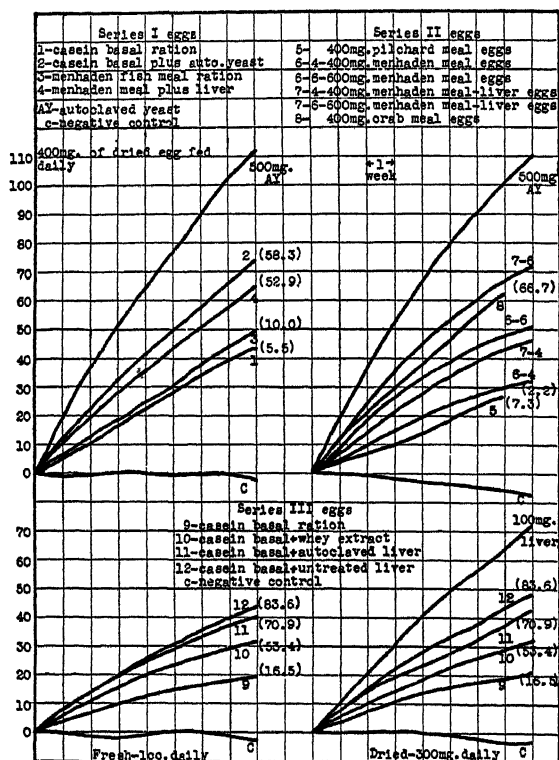


Fig. 4 The effect of the ration of the hen on the vitamin G content of the eggs. The figures in parentheses following the growth curves represent the hatchability (per cent of fertile) of the eggs from the same pens. In series I, five rats were included in the negative and positive control groups and eight in each of the egg groups. Series II contained from six to eight rats on each egg level supplement and four and nine in the positive and negative control groups, respectively. Ten rats were included in each of the groups in series III except the 100 mg. liver group, which contained four.

yellow corn, 58; ground wheat, 20; wheat bran, 5; Argentine casein, 12; steamed bone meal, 3; salt, 1, and cod liver oil, 1. This ration is known (unpublished results) to be deficient in vitamin G for chicks and leads to the development of a leg

disorder, as described by Norris and associates ('30) and Bethke et al. ('31), in a variable percentage of the birds. Beginning with the second week, the shell-free, hard-boiled eggs from the different pens of hens were fed daily for either 6 or 7 weeks.

In the first experiment the eggs from some of the same pens of birds as used in the series II rat studies (fish meal rations) were fed for 6 weeks to groups of twenty-five chicks each. There was also included for comparison one group of chicks on the unsupplemented basal ration. The results, presented in table 1, show that the eggs from the different

TABLE 1

*Effect of feeding different fish meal rations to hens on the vitamin G content of the eggs for chicks*

EGG SUPPLEMENT	TOTAL BASAL RATION PER CHICK	TOTAL EGG PER CHICK	AVERAGE WEIGHT OF CHICKS	CHICKS SHOWING LEG DISORDER	CHICKS WITH LEG DISORDER RECOVERED	HATCH- ABILITY OF EGGS <sup>1</sup>
	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>no.</i>	<i>no.</i>	<i>%</i>
None	556.5	....	173.1	15	3	....
Menhaden fish meal	528.4	215.8	232.5	21	6	2.2
Pilchard fish meal	569.0	216.0	268.2	18	8	7.3
Pilchard fish meal plus 5% dried skimmilk	718.6	226.0	321.1	12	6	46.0
Menhaden fish meal plus 5% dried liver	787.5	225.0	405.8	13	12	....
Crab scrap meal	797.2	216.0	423.4	2	2	66.8

<sup>1</sup> On the basis of fertile eggs.

pens of hens varied in their vitamin G content. The eggs produced on the menhaden and pilchard fish meal rations were less potent in vitamin G, as determined by growth and the prevention of the leg disorder, than eggs produced on similar rations supplemented either with 5% commercial liver meal or 5% dried skimmilk. It is also significant to note that, as in the rat experiments, the higher the hatchability of the eggs the greater was their vitamin G potency. The experiment was, in part, repeated by comparing the eggs from the menhaden fish meal and menhaden fish meal-liver meal pens, with results very similar to those obtained in the above trial.

The second experiment involved a comparison of the eggs from the same pens of hens as in the series III rat trials. The eggs were fed for 7 weeks to groups of eighteen chicks each. There were included for control purposes one group of chicks on the unsupplemented basal ration and another on the basal ration with 3% of dried pork liver. The liver was incorporated in the basal ration when egg feeding was begun, and the protein content adjusted to that of the basal ration by decreasing the amount of casein. The results, table 2, again show that the vitamin G value of the eggs was affected

TABLE 2

*Effect of adding vitamin G supplements to the ration of hens on the vitamin G value of the eggs produced for chicks*

EGG SUPPLEMENT	TOTAL BASAL RATION PER CHICK	TOTAL EGG PER CHICK	AVERAGE WEIGHT OF CHICKS	CHICKS SHOWING LEG PARALYSIS	CHICKS WITH LEG PARALYSIS RECOVERED	HATCH- ABILITY OF EGGS <sup>1</sup>
	gm.	gm.	gm.	no.	no.	%
None	577.6	....	184.6	13	0	....
Casein basal ration	681.8	220.0	260.4	14	3	16.5
3% autoclaved dried liver	891.0	203.0	369.2	12	5	70.9
Extract of dried whey $\approx$ 10%	679.2	204.0	275.6	16	6	53.4
3% dried liver	1002.7	190.0	449.3	2	1	83.6
Basal ration plus 3% dried liver	1393.0	....	574.4	0	0	....

<sup>1</sup> On the basis of fertile eggs.

by the ration of the hen. The inclusion of either autoclaved or untreated dried pork liver in the basal ration of the hens caused the production of eggs with a greater vitamin G content; which was correlated with the hatchability of the eggs. The results obtained with the eggs from the whey extract ration are not in accord with the results procured on the other rations or the rat assay. The chick experiment revealed no difference between the basal ration and basal ration-whey extract eggs; whereas the rat assay and incubation results showed the latter eggs to contain more vitamin G and to hatch better than those produced on the basal ration. A second comparison of the eggs from the basal and whey extract

rations with chicks did not show a significant difference in vitamin G content. We have no explanation to offer for this apparent inconsistency in the results with rats and chicks, unless we were not measuring the same factor in the eggs with the two different species.

Aside from the above exception, the results of the rat and chick assays on the eggs are in good agreement and show that the vitamin G intake of the hen affects the potency of her eggs in this factor. The results also show that embryonic development (hatchability) is closely associated with the vitamin G content of the egg. Apparently vitamin G is essential for embryonic development and unless adequate amounts of this factor are present in the egg, the embryo will die prematurely.

It was observed in preparing the eggs for rat feeding that there was a noticeable difference in the color of the egg white from the different pens. Invariably, the egg whites from those pens which received either autoclaved yeast, dried whey, or liver in their rations showed a distinctly more yellowish green color than the whites from the eggs on the unsupplemented basal rations. This observation suggests that the differences noted in hatchability and in vitamin G content of the eggs might be due to flavin, which has recently been shown to be associated with vitamin G. Experiments are in progress to obtain further information on this point.

#### SUMMARY

A study of the distribution of vitamins B and G in eggs produced on a good practical ration showed that vitamin B is present in the egg yolk and not in the egg white and that vitamin G is found in both the white and yolk.

Experiments with rats and chicks showed that the ration of the hen affects the vitamin G content of the eggs produced. The inclusion of dried skimmilk, dried whey, autoclaved yeast, dried liver, or certain fish meals in rations low in vitamin G resulted in the production of eggs of increased vitamin G content.

Evidence is presented which shows that embryonic development of the chick (hatchability) is related to the vitamin G content of the eggs.

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# THE EFFECT OF POLYNEURITIS IN CHICKS UPON THE IN VIVO RATE OF REMOVAL OF PYRUVATE INJECTED INTRAVENOUSLY<sup>1</sup>

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ONE FIGURE

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Our work with chicks, and the investigations of Peters and co-workers with pigeons, indicate that vitamin B(B<sub>1</sub>) is concerned with the metabolism of pyruvic acid. Most of the evidence in support of this view is based upon in vitro studies of respiration of tissues from polyneuritic birds. Peters and Thompson ('34) found that pyruvic acid accumulated during the respiration in lactate of brain from polyneuritic pigeons. With normal pigeon brain under similar conditions pyruvic acid did not accumulate. Vitamin B (B<sub>1</sub>) added to avitaminous pigeon brain respiring in lactate or pyruvate increased the oxygen uptake. We have shown that in avitaminous chick brain additions of vitamin B had no effect upon respiration in lactate (Sherman and Elvehjem, '36 a, '36 b) but that with pyruvate as substrate, similar additions of vitamin B increased the oxygen uptake of avitaminous cerebrum and kidney.

Although there is good evidence to show that there is a faulty metabolism of pyruvic acid in avitaminous pigeon and chick tissues in vitro, there is insufficient evidence of a failure in the in vivo metabolism of pyruvate in avitaminosis B to

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justify the conclusion that the syndrome of polyneuritis is directly caused by an accumulation of pyruvic acid. Analyses of pigeon brain immediately after the death of the animal have given no indication of a rise in pyruvic acid content (Thompson and Johnson, '35), but Kinnersley and Peters ('29) found an increase in the lactic acid content of avitaminous pigeon brain immediately after death. The failure to detect a rise in the pyruvic acid content of freshly-removed avitaminous tissues may be due, as Thompson and Johnson ('35) point out, to its diffusion from the tissues into the blood stream, since a threefold rise in blood pyruvate of polyneuritic pigeons and more than a twofold rise in the pyruvate content of polyneuritic rats was detected. Pyruvic acid has recently been isolated from the blood of B deficient pigeons (Johnson, '36) in the form of the 2:4 dinitrophenylhydrazone.

Similar studies in our laboratory have given no indication of a rise in either the lactic acid (method of Friedemann and Graesser, '33) or the pyruvic acid (method of Clift and Cook, '32) content of cerebrum or heart in polyneuritic chicks. Nor have we been able to detect a consistent rise in the pyruvate content of avitaminous chick blood. However, in the feces of polyneuritic chicks we have found a considerable rise in bisulphite-binding substances. But before these bisulphite-binding substances can be called pyruvic acid, isolation and characterization studies will be necessary.

Since avitaminous tissues are unable to metabolize pyruvate in contrast to normal tissues, it seems probable that in polyneuritis the excretory organs must have an increased load in order to prevent as long as possible an excessive accumulation of pyruvate in the blood and tissues. Sodium pyruvate was therefore injected intravenously into normal and avitaminous chicks and the rate of its removal was determined. Our results show that injected pyruvate disappears from the blood stream of normal chicks so rapidly that no significant increase could be detected 1 minute after injection of amounts of pyruvate sufficient to produce a great theoretical increase in blood pyruvate. In polyneuritic



chicks, on the other hand, injection of sodium pyruvate produced a sharp increase in blood pyruvate which slowly returned to the normal level.

#### METHOD

Large normal and polyneuritic chicks weighing from 200 to 400 gm. were used in these studies. Fresh vacuum-distilled pyruvic acid was adjusted to pH 7.3 with NaOH and made into a solution containing 40 mg. pyruvic acid per cubic centimeter. Large veins under each wing were exposed for injection and blood removal. A 27-gauge hypodermic needle attached to a hypodermic syringe containing the solution of sodium pyruvate was inserted into the vein under one wing and a measured amount of solution carefully injected. After a few seconds had elapsed following the injection to allow the blood to carry all of the injected pyruvate from the site of injection back to the heart, the needle was removed. Blood samples were then taken at frequent time intervals from the corresponding vein on the opposite wing with a capillary pipette calibrated to contain 0.2 cc. The blood samples were discharged into centrifuge tubes containing 9.8 cc. of 4% trichloroacetic acid. The thin film of blood clinging to the inner wall of the pipette was removed with the trichloroacetic acid. Following this technic it was usually possible to obtain the first blood sample from 1 to 2 minutes after the time of injection. The method is somewhat crude, especially in the measurement of the exact time of the injection and the subsequent sampling of blood, since a rapid flow of blood is not always obtained. The time intervals indicated are accurate to within only  $\pm\frac{1}{2}$  minute. Pyruvate analyses here recorded were made by the method of Clift and Cook ('32) according to the technic described in an earlier publication (Sherman and Elvehjem, '36 a, '36 b).

For the comparison of the bisulphite-binding capacity of normal and avitaminous chicks, larger samples (2 to 3 cc.) were collected in a beaker from the neck of the decapitated chick and quickly poured into a centrifuge tube containing

trichloroacetic acid. The tube was immediately stoppered and weighed. Samples of feces (about 1 gm.) were removed from the cloaca and treated with trichloroacetic acid in the same manner as the blood samples. A few analyses were also made on the contents of the large intestine and are included for comparison.

### RESULTS

The values obtained for the bisulphite-binding capacity of blood and feces from normal and polyneuritic chicks are given in table 1. There is no apparent increase in the bisulphite-

TABLE 1

*Bisulphite-binding substances in the blood and feces of normal and avitaminous chicks. Calculated as milligrams pyruvic acid/100 gm.  
(1 cc. N/200 I = 0.22 mg. pyruvic acid)*

BLOOD		FECES	
Normal	Avitaminous	Normal	Avitaminous
11.6	14.2	From large intestine	
19.0	12.2	61	68
17.8	27.8	59	82
15.6	10.9	From cloaca	
13.9	12.1	115	304
	9.3	116	606
		141	282
		139	264
			288
15.6	Average 14.4	128	Average 349

binding capacity of the blood of the B deficient chick over the normal level. In chicken feces, on the other hand, a rise in bisulphite-binding substances was found in avitaminosis B. In the cloaca, into which the urinary excretion flows, there was nearly a threefold rise in bisulphite-binding substances. A few samples of the contents of the large intestine above the cloaca were analyzed for bisulphite-binding capacity and did not show such a large increase in the polyneuritic chicks.

The effect of the intravenous injection of various amounts of sodium pyruvate upon the blood pyruvate of normal and

avitaminous chicks is given in figure 1. The curves immediately after injection are dotted because no blood samples could be taken during this time, and the immediate effect of the injection upon the pyruvate level of the blood is unknown. With all levels of pyruvate injected a pronounced rise in the blood pyruvate of polyneuritic chicks is apparent in the first sample after the injection (2 or 3 minutes). Succeeding samples show that the high initial pyruvate level drops off rapidly at first and then slowly approaches the normal level.

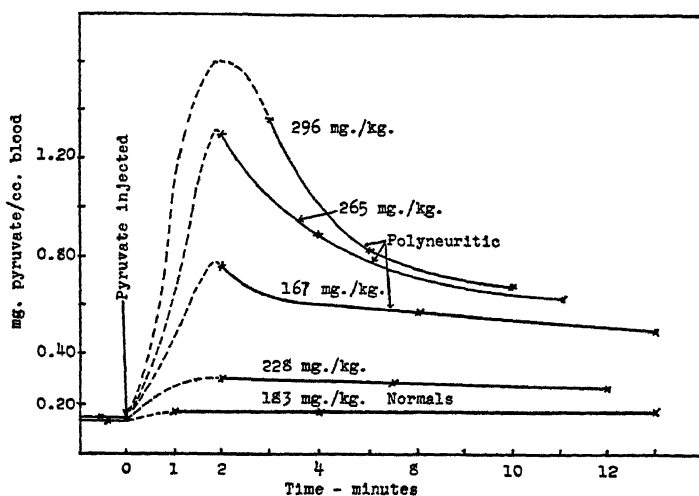


Figure 1

Intravenous injections of comparable levels of pyruvate into normal chicks produce only a very slight rise in blood pyruvate. The first sample was taken as soon as possible after the injection in order to detect, if possible, an increase of short duration, but none was found.<sup>2</sup>

Such a rapid removal of pyruvate from normal blood suggested the possibility that blood itself might be able to metabolize pyruvic acid. Normal blood and avitaminous blood with and without added vitamin B were tested *in vitro* for their ability to metabolize pyruvate. Sodium pyruvate

<sup>2</sup> We wish to thank Mr. V. R. Potter for his assistance in making injections.

was added to Ringer's phosphate + pyrophosphate buffer pH 7.3, and made isotonic by the addition of NaCl. This solution containing 4.14 mg. pyruvic acid per cubic centimeter was mixed with equal volumes of the three samples of blood and incubated for 1 hour at 37°C. and vigorously shaken with air every 5 minutes. During this period of incubation 1 cc. samples were removed from each tube at frequent intervals for pyruvate analysis. Reference to table 2 shows that pyruvic acid is not metabolized by either normal blood, avitaminous blood, or avitaminous blood with added vitamin B.

TABLE 2

*Aerobic removal of added pyruvate by normal and avitaminous chick blood in vitro. Results are expressed in milligrams. Pyruvic acid/cubic centimeter of diluted blood. Time intervals are given in minutes after mixing the blood with the pyruvate*

TUBE	BLOOD	ADDITIONS	BLANK ON BLOOD MG./CC. WHOLE BLOOD	INITIAL CONCENTRATION	CONCENTRATIONS AT LATER TIME INTERVALS, MINUTES						
					6	12	18	24	33	41	56
1	Normal	Pyruvate	0.15	2.12	2.12	2.12	2.16	2.14	2.12	2.14	2.12
2	Avitaminous	Pyruvate	0.13	2.13	2.16	2.16	2.17	2.20	2.18	2.19	2.18
3	Avitaminous	Pyruvate + vitamin B	0.13	2.13	2.18	2.22	2.20	2.18	2.22	2.20	2.18

Each tube contained 5 cc. of blood and 5 cc. of Ringer phosphate-pyrophosphate buffer to which sodium pyruvate was added (4.14 mg./cubic centimeter). Five-tenths cubic centimeter (12.5 gamma) of B replaced an equal volume of buffer in tube 3.

## DISCUSSION

Our results indicate that in chicks polyneuritis does not produce a rise in blood bisulphite-binding substances. The values for normal and polyneuritic chicks are comparable to results reported by Thompson and Johnson ('35) for avitaminous pigeon blood. But they found the normal level in pigeon blood to be only one-third the avitaminous level. It appears that the normal level of bisulphite-binding substances in the blood of chicks on a natural grain ration so closely approaches the renal threshold that any further increase arising from a faulty metabolism of pyruvic acid in the

avitaminous tissues results in its excretion since we have found a threefold increase in the excreta of polyneuritic chicks. As yet we have not attempted to characterize the compounds present in normal and avitaminous chick blood and feces responsible for their bisulphite-binding capacity. The results of Thompson and Johnson ('35) indicate that the bisulphite-binding compounds of normal pigeon blood are not pyruvic acid. Similar studies upon the nature of the bisulphite-binding substances of chick blood and feces should be undertaken to determine if there is a change in polyneuritis.

Injections of pyruvate show that pyruvic acid is more rapidly removed from normal blood than from avitaminous blood. Since this difference in the rate of removal cannot be attributed to changes in the blood metabolism of pyruvic acid in polyneuritis, it must be due to either a lowered rate of excretion or to faulty tissue metabolism. In normal chicks injected pyruvate is rapidly removed from blood because two channels of removal are at hand: 1) Absorption from the blood by the tissues where it is metabolized. 2) Passage into the excreta. But in avitaminosis B, because of faulty tissue metabolism of pyruvate, the rate of removal is largely dependent upon the excretory process. The time necessary for all the blood to be filtered through the kidneys is sufficiently great to permit the detection of a rise in blood pyruvate following its injection.

#### SUMMARY

1. The bisulphite-binding substances of normal and B avitaminous chick blood and feces have been investigated. Intravenous injections of sodium pyruvate were made in normal and polyneuritic chicks, and its rate of removal from the blood determined.

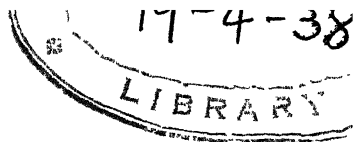
2. There is no rise in the bisulphite-binding substances of the blood of chicks in avitaminosis B, but the excreta of polyneuritic chicks contain increased amounts of bisulphite-binding substances.

3. Pyruvate injected intravenously is very rapidly removed from the blood of normal chicks. A slow rate of removal was found in polyneuritic chicks.

4. These phenomena are consistent with the view that in polyneuritis there is a failure in the tissue metabolism of pyruvic acid.

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## FACTORS INFLUENCING THE INCIDENCE OF DIETARY HEMORRHAGIC DISEASE IN CHICKS

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TWO FIGURES

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Evidence for the existence of a new fat-soluble vitamin required by the chick for the prevention of hemorrhagic tendencies and increased blood clotting time has been reviewed in a previous paper (Almquist and Stokstad, '35). Examples of chicks afflicted with hemorrhages as a result of a deficiency of this vitamin are shown in figures 1 and 2. The anti-hemorrhagic vitamin has been concentrated to a state of high potency, about 2 parts of the concentrate per million parts of diet being adequate (Almquist, '36 a). The purity of this concentrate has been increased about four times by distillation under high vacuum (Almquist, '36 b).

It appeared necessary, for the purpose of standardizing assay procedures for the anti-hemorrhagic vitamin, to study certain possible causes of variation in the incidence of this disease. That the exposure of ingredients of the diet to the action of the microorganisms can cause the development of anti-hemorrhagic activity in such materials has already been shown (Almquist and Stokstad, '35). A possible similar synthesis of this factor in the fecal matter of chicks has been investigated. In addition, the transfer of the anti-hemorrhagic vitamin from the diet of the hen to the chick has been studied.

## METHOD AND RESULTS

White Leghorn chicks were kept in electrically heated, wire-floored, all metal brooders. The number of chicks in each experimental group varied from ten to fifteen. The basal diet used was as follows:

Ether extracted fish meal	17.5
Ether extracted dried brewer's yeast	7.5
Salt plus small amounts of cupric and ferrous sulphates	1.0
Cod liver oil	1.0
Ground polished rice	73.0

This diet contains approximately 18.2% protein, 2.6% ether extract, 4.9% ash, 0.9% calcium and 0.5% phosphorus. When supplemented with the anti-hemorrhagic vitamin, it allows chicks to maintain good health and to grow at normal or better rates. When not so supplemented, this diet causes severe hemorrhagic symptoms within 3 weeks time and heavy mortality, but the growth rate of chicks is not directly affected until the hemorrhagic condition becomes severe.

Droppings from chicks 4 weeks old which had received only the basal diet were collected at 24-hour intervals and dried by immersion in several changes of ethyl alcohol. The dried droppings were then extracted continuously with ethyl ether. The alcohol and ether extracts were mixed and diluted with a large volume of water. The ether layer was drawn off and concentrated.

When incorporated in the basal diet, this fecal extract was found adequate as a source of the anti-hemorrhagic vitamin at a level as low as the equivalent of 5% of dry fecal matter in the diet, and was found marginal at a 1% equivalent level. The extracted fecal residue gave no evidence of protection at a 5% level. The results of this experiment proved that the anti-hemorrhagic vitamin was present in appreciable quantities in the droppings of chicks which were given a diet free of the vitamin. The vitamin was evidently synthesized by bacterial action either after the droppings were voided or within the lower portions of the digestive tract where absorption could not take place readily, or both.



A second collection of droppings from a similar group of chicks was made by allowing them to fall directly into a tray containing 1% phenol in aqueous solution in order to inhibit further bacterial action. The droppings were extracted as before. The final ether solution was washed several times with dilute sodium hydroxide solution and with water.



Fig. 1 Subcutaneous hemorrhage on right wing. Chick 3 weeks old. Weight 170 gm.



Fig. 2 Intra-muscular hemorrhages on inside of right leg, left side of breast and left shoulder. Chick 4 weeks old. Weight 245 gm.

As a source of the anti-hemorrhagic vitamin, the ether solution was found adequate at an equivalent level of 8%, marginal at 4% and inadequate at 2%. Presence of the vitamin in this preparation was thus demonstrated, however, the potency was apparently lower than that of the first extract. Because of the high stability of the anti-hemorrhagic vitamin it is unlikely that any of it was destroyed by this treatment.

To obtain evidence on the possible transference of the anti-hemorrhagic vitamin from the diet of the hen to her chicks, eggs from eight pens of laying hens having different levels of dried alfalfa in their diets were hatched. The various diets given the hens approximated normal egg production diets except for the omission of dried alfalfa in certain cases. No supplemental green feed was used in any pen. The chicks from these pens were fed only the basal diet. Records were kept of the number of days lived by each chick and of incidence of hemorrhagic symptoms. The data obtained are given in table 1.

TABLE 1

PEN	PERCENTAGE OF DRIED ALFALFA IN DIET OF HENS	PERCENTAGE INCIDENCE OF HEMORRHAGES IN CHICKS			PERCENTAGE MORTALITY OF CHICKS			AVERAGE NUMBER OF DAYS LIVED PER CHICK
		Weeks			Weeks			
		4	8	12	4	8	12	
141	0	77	94	94	82	100	100	24.5
142	10	13	22	39	35	45	70	50.7
143	0	35	65	94	47	70	100	34.8
144	2½	12	38	43	19	67	76	50.3
145	0	40	80	80	50	100	100	31.3
146	5	13	33	60	13	40	73	55.6
147	0	45	72	83	61	83	100	25.2
148	5	24	60	60	28	75	80	42.6

In this experiment dried alfalfa which is rich in the anti-hemorrhagic vitamin was the principal source of this vitamin given the hens. There was a distinct relation between the presence of this supplement in the diet of the hens and the results obtained with their chicks. In all cases, where alfalfa was fed to the hens the chicks developed hemorrhagic symptoms less readily and survived for a significantly greater length of time. In fact, several birds in these four lots were still alive at the conclusion of the experiment. The variation in the level of alfalfa from 2½ to 10% did not seem to affect the results.

Presence of the anti-hemorrhagic vitamin in egg yolk was demonstrated by feeding hard boiled whole egg yolk at the rate of one-fifth yolk per chick per day. This quantity gave

ample protection. The egg albumen, similarly fed, gave no protection. In a previous paper (Almquist and Stokstad, '35) we reported that the vitamin was not present in the non-saponifiable fraction of yolk fat. It is now known that the vitamin was probably destroyed during the saponification procedure (Almquist, '36).

The vitamin does not seem to be stored in the livers of young chickens (about 8 weeks of age) raised on normal diets containing 5% of dried alfalfa. Liver tissue from such birds was dried in vacuum and fed at 5 and 10% levels as a supplement to the basal diet. In both cases, no appreciable anti-hemorrhagic activity was found.<sup>1</sup>

#### DISCUSSION

The results on assays of fecal material indicate that chicks used in assays for the anti-hemorrhagic vitamin must not have access to their droppings. The results also indicate that the vitamin may be synthesized, presumably by bacterial action, within the intestinal tract of the chicks but evidently in a region where absorption does not take place. Such synthesis may, however, afford an explanation for cases of spontaneous recovery which we have frequently observed if, under certain conditions, some absorption of the synthesized vitamin may take place. It is also possible that the birds may occasionally consume fecal material before it passes through the wire mesh floor.

Droppings collected under antiseptic conditions appeared to have less potency than those collected at 24-hour intervals, indicating that appreciable further synthesis of the vitamin may have occurred in the latter.

That the diet of the hen influences the incidence of the hemorrhagic syndrome in her chicks is also a demonstrable fact. It has been found desirable to restrict the diet of hens producing chicks for this work to a minimum quantity of dried greens

<sup>1</sup> Since the preparation of this manuscript there has appeared a paper by H. Dam and F. Schonheyder, *Biochem. J.*, vol. 30, p. 897, 1936, in which similar results with liver tissue from young normal chicks are described.

(2.5%) which will permit reasonably satisfactory hatchability and to allow the hens to receive no fresh green feed. Chicks from such a source have given satisfactory response to their diets within 2 weeks.

Precautions must be observed to avoid bacterial action on any or all portions of the diet. For example, feed carried by the chicks to the water troughs must not be allowed to accumulate there and the troughs must be cleaned frequently.

It has been found necessary for uniform results to extract with ether the fish meal and yeast used in the basal diet. When casein is employed it must also be extracted. One of the chief reasons for the use of rice in the basal diet is that it may be obtained in a high state of cleanliness and uncontaminated with weedy or green extraneous plant tissue or diseased kernels. It is free of the anti-hemorrhagic vitamin since extraction of the rice does not increase the severity of the disease.

In certain cases we have obtained indications that alfalfa dust in the atmosphere may influence the results of assays.

Such precautions as have been mentioned are a result of the fact that comparatively very small quantities of the anti-hemorrhagic vitamin are required by the chick.

#### SUMMARY

1. The anti-hemorrhagic vitamin of the chick is present in fecal matter of chicks receiving none of this vitamin in the diet. It is probably synthesized to some extent within the lower portions of the intestinal tract of chicks and to a further extent in droppings allowed to stand for 24 hours. Chicks employed in studies of this vitamin must not have access to their droppings.

2. The anti-hemorrhagic vitamin is transferred from the diet of the hen to her chick. It is advisable to restrict the diet of hens producing chicks for studies of this vitamin to a minimum level of substances rich in the anti-hemorrhagic vitamin.

3. The anti-hemorrhagic vitamin is present in egg yolk but not in egg albumen. Liver tissue from young normal chicks contains no appreciable quantity of the anti-hemorrhagic vitamin.

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# METABOLIC STUDIES OF ESKIMOS IN THE CANADIAN EASTERN ARCTIC

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The data reported here were obtained in 1935 during the Canadian Government Eastern Arctic Patrol on the R. M. S. Nascopie. The examinations included Eskimos in Hudson Bay and Strait, the more northerly parts of Baffin Island and in Devon and Ellesmere Islands as far north as 76° 12' north latitude—828 nautical miles from the Pole.<sup>1</sup> For reasons stated in the clinical report of this investigation (Rabinowitch, '36) and, as will presently be seen, some of the data are unavoidably fragmentary and are reported for purposes of record and future reference only.

## BLOOD CHEMISTRY

The blood analyses include total non-protein nitrogen, urea nitrogen, creatinine, uric acid, amino acid, sugar, chlorides and lipoids. The lipoid studies form a subject matter of another report.

*Preparation of materials.* None of the tests was completed during the voyage. The work then was confined to collection of the bloods and the necessary treatment in order to preserve them. Folin-Wu (tungstic acid) filtrates were prepared for determination of total non-protein nitrogen, urea nitrogen, creatinine, uric acid and amino acids. To each filtrate were

<sup>1</sup> A portion of the material on the investigation of which this report is based was collected by Dr. C. C. Birchard in Hudson Strait and on the Quebec shores of Hudson Bay.

added a few drops of toluol and all materials were kept cold during the entire voyage. As a further precaution against deterioration, the pH of each filtrate was determined prior to any of the chemical analyses and, without exception, every filtrate was found definitely acid; the pH values ranged between 3.7 and 4.4. The pH determinations were made with the Hellige Standard Model Hydrogen Ion comparator with permanent colored-glass standards.

All chloride determinations were made upon plasma. In each case, the blood was centrifuged as soon as possible after it had been collected and the separated plasma was placed in a tightly corked tube and kept in the refrigerator of the ship during the entire voyage. As all of the laboratory work had to be done on the ship, in a few cases, owing to tide conditions, some hours had elapsed before the bloods could be centrifuged. It is well recognized that unless the plasma and red blood cells are immediately separated from each other after blood has been collected, the concentration of chlorides in the plasma tends to decrease owing to ionic mobility. This possible source of error will again be referred to in the interpretation of the data.

Sufficient quantities of the Folin-Wu filtrates were available for the determinations of blood sugars by a number of methods (Shaffer-Hartmann, Folin-Wu, etc.). The blood sugars to be discussed here, however, were obtained with a slight modification of the Myers-Benedict picric acid method. The reason for this selection will be dealt with in the discussion of the results.

A summary of all of the blood analyses is given in table 1. For purpose of brevity, the number of analyses and maximum, minimum and average values only are recorded.

*Discussion of results. Non-protein nitrogen.* Bloods of forty-six Eskimos were examined; but in five, after the other constituents had been determined, there were insufficient quantities of the tungstic acid filtrates left for estimation of amino acids. It will be observed that, with the exception of uric acid, though the minimum values of the different constituents are within the generally accepted limits of normality,



the maximum and average values are high. Heinbecker ('28) reported a number of total non-protein nitrogen values in three Eskimos which indicated no retention of nitrogenous products in the blood from the habitual high protein diets; the values ranged between 29 and 43 mg. In one analysis, a very high value—112 mg.—was found; but, in this case, the author suggested that the result was probably due to faulty precipitation of the blood proteins. The observations was also made that by the time the filtrates had been returned to St. Louis for examination all had acquired slight growths of molds. In none of our filtrates was there any suggestion of incomplete

TABLE 1  
*Blood chemistry of Eskimos (Canadian Eastern Arctic)*

CONSTITUTION	NUMBER OF ANALYSES	MILLIGRAMS PER 100 CC.		
		Maximum	Minimum	Average
Non-protein nitrogen	46	64	18	42.0
Urea nitrogen	46	45	14	28.3
Creatinine	46	1.91	1.00	1.55
Uric acid	46	5.26	1.18	3.38
Amino acids	41	10.8	4.0	7.66
Sugar	13	151	110	132
Chlorides (as NaCl)	34	634	560	603

precipitation of proteins and none had any molds. The importance of excluding molds was shown in one of our experiments in which the non-protein nitrogen was determined before and 2 weeks after exposure of a Folin-Wu blood filtrate to room temperature. After the molds had been removed by filtration, 29 only of the 40 mg. of non-protein nitrogen found originally were recovered from the filtrate. Molds, therefore, can account for an appreciable amount of non-protein nitrogen. Heinbecker, however, has the impression that his findings included molds plus filtrate (personal communication).

Impairment of kidney function does not explain our high values. The urinalyses show that these Eskimos were remarkably free of albuminuria and there were no clinical signs to suggest renal disease otherwise (Rabinowitch, '36). These

high values are apparently due to the enormous quantities of meat which these Eskimos eat. When food is plentiful, a healthy adult may eat 5 pounds or more of meat a day. That this is the correct explanation is also suggested from the urinary nitrogen data (see nitrogen metabolism). Meat is often eaten in a putrified state. This, it would appear, is the explanation of the high amino acid values (proteolysis).

*Plasma chlorides.* In table 1 are also recorded the concentrations of chlorides found in the bloods (plasma) of thirty-four Eskimos. It will be noted that the maximum and average values (expressed as NaCl) are high, in spite of the fact that these Eskimos have no salt in their diets other than that naturally present in the food. As stated, owing to tide conditions, the bloods, in some cases, could not be centrifuged immediately. Therefore, since failure to separate plasma from cells immediately tends to decrease rather than increase the chloride content of plasma, some of the actual chloride concentrations were probably still higher than those found.

#### URINARY CHLORIDES

Opposed to the high concentrations of the chlorides in the bloods are the extremely low concentrations of chlorides in the urines. This is shown in table 2. It will be noted that the concentrations (also expressed as NaCl) which were found in twenty-two individuals ranged between 0.02 and 0.26% only. Unfortunately, owing to the short time spent at each port during the voyage, it was not possible to collect 24-hour samples of urine, in order to determine total excretions. These Eskimos drink large quantities of water, but dilution of the urines does not appear to be the explanation of these low concentrations for a number of reasons. First, a large part of the ingested water is eliminated by the skin; these people sweat very profusely. This should tend to increase rather than decrease the urinary concentrations of NaCl. That the volumes of urine are no greater among these Eskimos than of people elsewhere is shown by the data reported by Heinbecker ('28, '31, '32). Of forty-six 24-hour specimens, the volumes ranged between

473 and 2995 cc. The average was 1285 cc. Opposed also to undue dilution in our cases are color and specific gravity. None of the urines was pale and the densities ranged between 1.010 and 1.024. The average specific gravity of all of the twenty-two specimens was 1.015. The latter is undoubtedly low compared with the average specific gravity of urine of healthy people elsewhere. Diet is, however, a factor. The specific gravity of urine depends not only upon the amount,

TABLE 2

*Sodium chloride and nitrogen concentrations of urines of Canadian Eastern Arctic Eskimos*

PLACE	PER CENT		PLACE	PER CENT	
	NaCl	N		NaCl	N
Port Burwell	0.12	0.44	Pangnirtung	0.16	1.54
Port Burwell	0.24	0.59	Pangnirtung	0.20	1.75
Port Burwell	0.24	0.78	Pangnirtung	0.26	1.68
Port Burwell	0.20	0.46	Pangnirtung	0.16	0.91
Port Burwell	0.20	1.09	Pangnirtung	0.16	1.02
Wolstenholme	0.12	0.44	Pangnirtung	0.20	1.52
Wolstenholme	0.02	0.98	Pangnirtung	0.10	1.73
Georges River	0.24	0.82	Pangnirtung	0.10	1.57
Georges River	0.26	0.88	Pangnirtung	0.26	1.90
Craig Harbour	0.14	0.45	Pangnirtung	0.18	1.39
Craig Harbour	0.24	1.11	Pangnirtung	0.08	0.84
Maximum	0.24	1.11	Maximum	0.26	1.90
Minimum	0.02	0.44	Minimum	0.08	0.84
Average	0.18	0.73	Average	0.17	1.48

but also upon the types of solids. These Eskimos are essentially meat eaters and the end product of protein digestion—urea—does not yield as high a specific gravity as corresponding concentrations of NaCl, etc. Combining all of the data, therefore, there is reason to believe that the urinary output of sodium chloride in these Eskimos is very low and this fits in with the dietary habits of these people. They have as stated no salt other than that present naturally in the food which is chiefly meat.

Excretion of water by the skin should, as stated, tend to increase the concentration of NaCl in the urine. Therefore,

unless very large amounts of salt are excreted by the skin, these blood and urine data offer interesting speculation. Unlike urea and other constituents of urine, sodium chloride is a 'threshold' substance—its excretion depends to a large extent upon its concentrations in the blood; and like glucose and other 'threshold' substances, it probably has a threshold because life is incompatible without it. Are the high concentrations in the blood and low excretions in the urine, therefore, the result of a defensive mechanism; that is, an attempt to conserve sodium chloride by raising the renal threshold? It will be observed that the average value, namely, 0.603%, is well above the average renal threshold level of NaCl found among people elsewhere (Aitken, '29).

#### NITROGEN METABOLISM

In table 2 are also recorded the concentrations of nitrogen found in the above-mentioned twenty-two samples of urine. Since economy of material was not necessary all of the nitrogen determinations were made in duplicate with 5 cc. quantities and with the standard macro-Kjeldhal technic. The data are divided into groups, according to the dietary habits of the natives. In the first column are recorded urines of Eskimos whose diets, though largely native, consist of appreciable quantities of civilized man's foods; whereas, in the second column are recorded the data obtained at Pangnirtung, on Baffin Island, where flour, etc., is available about 2 months in the year only. The diets here are confined largely to the food materials of the environment (seal, etc.). As in the case of sodium chloride, limited significance must be attached to these findings, since high concentrations do not necessarily imply large excretions. The data are, however, very suggestive. It will be observed that the average concentration of nitrogen in the urines of the flesh-eaters was twice that of the natives whose diets are mixed. In one case, the concentration was very high, namely, 1.90%. High concentrations are, however, apparently not uncommon among these Eskimos, in view of the high protein contents of the diets and the relatively low urine

volume output. Thus, even during fasting, Heinbecker ('31) reported daily excretions of 18.90, 24.60 and 21.20 gm. of nitrogen. Since the volumes of urine in the corresponding experiments were 1400, 1380 and 1420 cc., the corresponding concentrations of nitrogen were 1.35, 1.73 and 1.49%, respectively.

The above findings fit in with the interpretation of the high concentrations of non-protein nitrogenous constituents found in the bloods (table 1). Inability to concentrate nitrogen in the urine is an early manifestation of impaired kidney function. Retention of nitrogen in the blood is a late result. These urinary nitrogen data, therefore, support the view that the high concentrations of non-protein nitrogenous constituents of the bloods were not due to impairment of kidney function, but to the habitual use of high protein diets. In our food analyses, for example, it was found that the seal meat contained 3.58% of nitrogen. One pound of such meat would, therefore, alone account for about 15 gm. of nitrogen in the urine and, as stated, when food is abundant, a healthy adult will eat much larger quantities. In these Eskimos, therefore, retention of nitrogen in the blood appears to be a physiological phenomenon.

#### COPPER METABOLISM

As was shown in the clinical report of this investigation (Rabinowitch, '36) there were a number of cases of polycythaemia among the Eskimos whose dietary habits and activities in general were largely native. Aside from their importance clinically, these cases are of interest with respect to the physiology of formation of haemoglobin. It is now generally believed that copper can, by some catalytic action, influence the rate of formation of haemoglobin and, in general, marine animals have much more copper than land animals. Were the excess quantities of haemoglobin in these cases of polycythaemia, therefore, due to excess intake of copper? This possibility was tested by estimation of the copper contents of the above-mentioned urines. The copper determinations were made by a method previously described by one of the writers

(Rabinowitch, '33) and, as with the urinary nitrogen, the data were divided into two groups according to the dietary habits of the natives. The findings are summarized in table 3. Assuming, therefore, that, as in the case of nitrogen, high concentrations may be taken to be due to large excretions, the data are suggestive. It will be observed that, as in the case of nitrogen (table 2) the highest concentration of copper in the urine and the highest average were found among the flesh-eaters. It should, however, be noted that the averages of both groups are within the limits of the concentrations of copper found in normal individuals elsewhere (Rabinowitch, '33). These averages alone do not, however, exclude copper as a factor in the production of the excess quantities of haemoglobin which were found in these Eskimos. Copper is also

TABLE 3

*Showing concentrations of copper (milligrams per liter) in urines of Eskimos*

	MIXED DIET	FLESH EATERS
Maximum	0.200	2.000
Minimum	0.050	0.075
Average	0.091	0.390

excreted—and probably to a large extent—by the intestines (bile) and as with other metals (lead, etc.) the urine may not reflect the concentrations in the tissues. 'Balance' experiments would have been helpful; but no preparations were made for this type of work; the polycythaemia was an accidental discovery during the clinical examinations.

#### CARBOHYDRATE METABOLISM

*Glycosuria.* In all eighty-eight urines were examined for sugar with Benedict's qualitative reagent, and reducing substances were found in three only. None of these substances, however, fermented with yeast. There was, therefore, no glucose in any of the specimens.

*Acetonuria.* The above-mentioned eighty-eight urines were also tested for acetone with the sodium nitro-prusside reaction. No acetone was found. All of the urines were preserved

in tightly corked test tubes. The corks were sealed and the tubes were kept cold throughout the entire voyage. Therefore, though there is the theoretical possibility of destruction of beta-oxy and beta-hydroxy-butyric acid, had any been present, there is no reason to doubt the reliability of the acetone data, since exposure of urine to as high a temperature as 38°C. does not affect total acetone values (Heinbecker, '28). However, according to the clinical findings (Rabinowitch, '36) there appears to be no mystery about the absence of ketosis. The Eskimo, in the Canadian Eastern Arctic at least, does not live upon as pure a protein-fat diet as is generally believed. There is a small supply of berries for about 2 months in the year. These Eskimos also relish the stomach contents of the caribou which contain carbohydrates throughout the year, though a large part is probably not utilizable as far as the human being is concerned (celluloses, hemicelluloses, hexosans, pentosans, etc.). One of the writers (IMR) was told that when these Eskimos catch a walrus, they immediately open the stomach and eat the clams which contain glycogen. The livers of all of the animals, with the possible exception of the bear, are eaten and these also contain glycogen. The skin of the whale and of the narwhal are both rich in glycogen and, as stated, when food is abundant, these Eskimos eat enormous quantities of meat which is largely eaten lean; except when food is scanty, blubber is not regarded as a relish. It is also of interest here to note that though some of the Arctic animals have enormous layers of blubber, the accumulation of fat in the musculature are not necessarily large. The meat, therefore, aside from the blubber, may be quite lean. Of the meats which we have examined that of the flipper of the white whale and of the walrus only were found to contain large quantities of fat—17.97 and 12.35%, respectively. The seal meat contained 4.97% of fat.<sup>2</sup> Seal, it should be noted, is regarded very highly, as it also supplies light and heat. It is, therefore, consumed in large quantities. When consideration is given to

<sup>2</sup>Krogh and Krogh are quoted as having found 6 to 10% of fat in seal (Heinbecker, '28).

these facts and also to the potential sugar production from protein (about 58%), it is obvious that the ratio of fatty acid to glucose is below the generally accepted level of ketogenesis. Owing to the short time available during this expedition, the calculations made of the diets of these natives must be accepted subject to modification; but the data suggest that when food is abundant, the average daily diet of the adult Eskimo in the Canadian Eastern Arctic consists, approximately, of 40 gm. of carbohydrate (including glycogen) 250 to 300 gm. of protein and about 150 gm. of fat (average FA/G = 1.2). This calculation, it will be observed, fits in with experiences with Greenland Eskimos. Shaffer ('21) cites calculations made by Krogh and Krogh of data collected by Rink, according to which the average diet of these Eskimos consists, approximately, of 54 gm. of carbohydrate, 135 gm. of fat and 282 gm. of protein (FA/G = 1.1).

As stated, these Eskimos have very little salt in their food. This alone might lead toward ketosis. Some of these Eskimos are, however, now perhaps, more exposed to the possibility of alkalosis rather than of acidosis, because of their practice in the use of 'banik.' 'Banik' is essentially a mixture of water, baking soda and flour and the majority of these people have no appreciation whatever of the necessary proportions of these materials in the preparation of this food. When finances permit, the diet may be confined largely to this 'banik' and the daily consumption, at times, may correspond to as much as 25 or more gm. of sodium bicarbonate.

*Blood sugar.* Though we also had Folin-Wu filtrates, the following observations, as stated previously, are based upon blood sugar data obtained with the Myers-Benedict picric acid method. There are a number of reasons for this selection. Since none of the sugar determinations could be made during voyage, consideration had to be given to the stability of glucose in blood filtrates. The stability of glucose in tungstic acid filtrates is uncertain. From many years of experience in this laboratory, we, however, know the stability of glucose in picric acid solutions. An equally important reason for the selection



of this method was comparability of data. In the interpretation of blood sugar time curves, it is generally accepted that normally the fasting blood sugar ranges between 0.08 and 0.12% and that the peak of the curve—the maximum hyperglycaemic response to ingestion of glucose—is below 0.18%. These standards, it should be noted, were originally derived from experiences with picric acid and not with copper reagents, the values with which are generally lower. Parenthetically, it may here be stated that, in the use of the picric acid technic, all precautions (Rabinowitch, '28) were taken to avoid artificially high values (test of purity of picric acid, etc.).

In table 1 are recorded thirteen sugar determinations of bloods collected at random and it will be observed that, though

TABLE 4  
*Blood sugar time curves of Eskimos*<sup>1</sup>

	NUMBER OF ANALYSES	BLOOD SUGAR (PER CENT)		
		Maximum	Minimum	Average
Before glucose	13	0.181	0.114	0.137
30 minutes after	11	0.250	0.175	0.210
60 minutes after	12	0.258	0.133	0.204
120 minutes after	11	0.192	0.119	0.149
150 minutes after	7	0.161	0.107	0.137

<sup>1</sup> Fifty grams of glucose.

the minimum value is within the generally accepted range of normality, the maximum and average values are high. However, in the interpretation of these data, consideration must be given to the fact that, as with the other blood constituents it was not always possible to collect all of the bloods in the fasting state, owing to the time of the day the ship arrived and the number of hours at the different ports. That the blood sugar of the Eskimo is, however, as a rule, higher than people elsewhere is suggested from the experiences with the thirteen blood sugar time curves shown in table 4. None of these tests was done unless it could be started in the fasting state. Again, for purpose of brevity, the number of tests and maximum, minimum and average values only are given for the different

points of the curves. Another reason, however, for the use of a composite curve, as the data show, is that it was not possible to obtain complete curves in all cases.

According to the average (composite) curve, it will be observed that the average blood sugar even in the fasting state was high. This also applies to the peak and to the other points of the curve, though it will be noted that, according to the minimum values, some of the points were within the generally accepted limits of normality. Assuming no technical error, these data, therefore, clearly indicate that these Eskimos had difficulty in utilizing glucose in pure form.

The above findings differ to some extent from the four curves reported by Heinbecker ('28). All of the latter were normal, though the Eskimos had followed their usual dietary habits prior to the examinations. Doctor Heinbecker kindly supplied one of the writers (IMR) with a description of the conditions under which these tests were made. All blood sugar determinations were made during the voyage and all reagents were tested before and after the determinations. A finding which is, however, difficult to reconcile with these normal curves is that "the normal height of the 12-hour fasting sugar of the Eskimo subjects is slightly higher than usually observed, averaging about 120 mg. %." This value, it should be observed, agrees very closely with the average found in our thirteen subjects, namely, 0.137%, when consideration is given to the methods used. Heinbecker made use of the Shaffer-Hartmann technic, the values with which are generally 10 to 15 mg. lower than with the picric acid technic. A blood sugar of 0.120% with the Shaffer-Hartmann method is, therefore, definitely high, since it represents the upper limit of normality with picric acid. It would, thus, appear that Heinbecker's Eskimos also had some difficulty to utilizing carbohydrates.

Urines were collected during these tests to correspond to the different points in the curves. There were, therefore, sixty-five specimens and sugar was found in six only. The amounts ranged between very faint traces and traces. In view of the

maximum blood sugars and the high average values at the 30- and 60-minute periods, it is obvious that some of these Eskimos also had a raised renal threshold for glucose.

#### RESPIRATORY QUOTIENTS

In table 5 are shown ten respiratory quotients obtained in the fasting state and it will be observed that five were lower than 0.7. Had these data been obtained elsewhere, they would have been discarded on the assumption that they were due to undetected technical errors. There were undoubtedly a number of disturbing factors. First, in spite of the excellent co-operation of the natives, conditions were not ideal for respiration work. The weather was cold and the subjects had to be examined in one of the holds of the ship. The latter was well

TABLE 5  
*Respiratory quotients of Eskimos obtained in the fasting state*

SUBJECT	FASTING STATE	SUBJECT	FASTING STATE
1	0.841	6	0.681
2	0.662	7	0.743
3	0.694	8	0.781
4	0.687	9	0.702
5	0.739	10	0.694

ventilated. Second, there was no opportunity to accustom the natives to the tests, because of the short time spent in any one port. However, lack of experience with the test should, because of over ventilation, tend toward artificially high rather than low quotients, owing to excess elimination of  $\text{CO}_2$ . No difficulties were encountered in the collection of the gas samples. All were preserved in tightly sealed tonometers and all analyses were done in duplicate. Two control samples—one from one of the writers—gave respiratory quotients of 0.830 and 0.813. Drowsiness, a possible cause of low quotients is excluded in these cases. None looked drowsy and drowsiness is incompatible with life in the Canadian Eastern Arctic. These people are very alert and all of the subjects were very interested in their tests. It is of interest here to note that

Heinbecker ('28, '31) observed similarly low quotients in Eskimos as early as the first day of fasting and the data fit in with those obtained by Higgins, Peabody and Fitz ('16) with fat-protein meals.

### BASAL METABOLISM

The basal metabolism of the Eskimo appears to be high; the average basal metabolic rate of ten subjects was plus 26%.

TABLE 6  
*Basal metabolism of Eskimos*

SUBJECT	SEX	AGE <sup>1</sup>	HEIGHT	WEIGHT <sup>2</sup>	BODY SURFACE	RESPIRATORY EXCHANGE		TOTAL R.Q.	TOTAL CAL. <sup>3</sup>	CAL. PER SQUARE METER	P.M.R. <sup>4</sup>
						CO <sub>2</sub>	O <sub>2</sub>				
			<i>inches</i>	<i>pounds</i>	<i>sq.m.</i>	<i>liters</i>	<i>liters</i>				
1	M	15	61.0	110	1.47	15.37	18.27	0.841	88.61	60.3	+ 31
2	M	45	63.0	132	1.62	12.52	18.91	0.662	88.61	54.7	+ 42
3	M	30	62.5	140	1.66	11.85	17.08	0.694	80.04	47.0	+ 19
4	F	20	60.0	100	1.39	9.52	13.86	0.687	64.94	46.7	+ 26
5	F	40	57.0	112	1.41	9.67	13.09	0.739	61.90	43.9	+ 22
6	M	55	64.0	136	1.65	10.29	15.11	0.681	70.80	42.9	+ 14
7	M	60	61.0	118	1.51	10.24	13.78	0.743	65.14	43.1	+ 18
8	F	30	63.5	111	1.51	11.90	15.24	0.781	72.78	48.2	+ 32
9	M	35	62.0	104	1.44	11.07	15.77	0.702	73.90	51.3	+ 30
10	M	50	65.0	148	1.74	12.16	17.52	0.694	82.10	47.2	+ 26
A.M.		38	61.9	121.2	1.54	11.46	15.86	0.722	74.88	48.5	+ 26

<sup>1</sup> Approximate (within 5 years).

<sup>2</sup> Adjusted for ounces.

<sup>3</sup> Calculated from calorific values of O<sub>2</sub> according to non-protein R.Q. Calorific value of 1 liter of O<sub>2</sub> at R.Q. less than 0.7 taken as at R.Q. = 0.7.

<sup>4</sup> Du Bois standards.

This is shown in table 6. There are, however, a number of possible errors inherent in the data. First, the basal metabolic rates were calculated from the respiratory quotients and it was assumed that the calorific value of a liter of oxygen with respiratory quotients lower than 0.7 was the same as at 0.7. There is also a possible error in the use of the Aub and Du Bois standards. These may not, and probably do not, apply to Eskimos. It should, however, be noted that with the

use of these low respiratory quotients, we have assumed minimum rather than maximum heat values. The true calorific values of the oxygen consumed may, therefore, have been greater than those used in the calculations. The true basal metabolic rates may, therefore, have been greater than those found.

A priori, one would expect these Eskimos to have high basal metabolic rates. The constant stimulation of the cold weather should, theoretically, tend toward increased muscle tone and basal metabolism is, to a very large extent, a function of active protoplasmic mass. These people are also, as stated, largely carnivorous in their dietary habits. There is, therefore, the added factor of the high specific dynamic action of proteins. A number of these individuals also had polycythaemia. This may also have been a factor, though according to Schaternikoff ('28) the ability of the blood to carry oxygen does not appear to be an important factor. In order, however, that the data may be subjected to further analysis when more is known of the variables mentioned, all of the details of the tests are recorded in the table. The data include age, sex, height, weight, body surface, consumption of oxygen and production of carbon dioxide.

#### METABOLISM AFTER INGESTION OF FAT

In table 7 are recorded two tests in which an attempt was made to determine the effects of ingestion of fat upon the respiratory quotient and metabolic rate. In each case, the fat meal consisted of 200 cc. of soya bean oil. It will be noted that respiratory quotients lower than 0.7 were observed here also, though in both subjects the quotients in the fasting state were definitely higher. Combining all of the data, it would, therefore, appear that in the Eskimo may be found the solution to the problem of conversion of fat to sugar. It is of interest to note that the increased heat production after each meal corresponded to the specific dynamic action of peoples elsewhere. As with the basal metabolic rates, however, all of the data are recorded in detail for any re-calculations which may be found necessary with a better knowledge of variables.

## SPECTROGRAPHIC ANALYSES

Because of a) the alleged absence of arteriosclerosis among Eskimos, b) the high incidence of arteriosclerosis among civilized people, c) the well-known harmful effects of lead upon the cardio-vascular system and d) the fact that small amounts of lead are almost invariably found in urines of perfectly normal individuals, particular attention was paid to lead in this investigation. Because it was suspected that minute

TABLE 7  
*Metabolism of Eskimos. Effects of ingestion of fat*<sup>1</sup>

PERIOD	RESPIRATORY EXCHANGE		R.Q.	HEAT PRODUCTION (CALORIES) <sup>3</sup>	
	CO <sub>2</sub>	O <sub>2</sub>		Total	Increase above basal
Experiment 1. Subject no. 5. Female. Age 40. <sup>2</sup> Height 57 inches. Weight 112 pounds. Body surface 1.41 sq.m.					
Fasting	<i>liters</i> 9.67	<i>liters</i> 13.09	0.739	61.90	%
1 hour after meal	9.92	14.53	0.683 <sup>4</sup>	68.09	10.0
2 hours after meal	9.87	14.95	0.660 <sup>4</sup>	70.05	13.2
3 hours after meal	9.52	13.49	0.706	63.21	2.1
Experiment 2. Subject no. 7. Male. Age 60. <sup>3</sup> Height 61 inches. Weight 118 pounds. Body surface 1.51 sq.m.					
Fasting	10.24	13.78	0.743	65.14	
1 hour after meal	11.42	15.62	0.731	73.63	13.0
2 hours after meal	10.64	15.05	0.707	70.52	8.2
3 hours after meal	8.94	14.58	0.613 <sup>4</sup>	68.32	4.9

<sup>1</sup> Two hundred cubic centimeters Soya bean oil.

<sup>2</sup> Approximate (within 5 years).

<sup>3</sup> Calculated from calorific values of O<sub>2</sub> according to non-protein R.Q.

<sup>4</sup> Calorific value of 1 liter of O<sub>2</sub> at R.Q. less than 0.7 taken as at R.Q. = 0.7.

traces only would be found, the urines were subjected to spectrographic rather than to chemical analysis. The spectrograms were made in the physics laboratories of McGill University by Dr. John S. Foster. No lead was found in any of the twenty-four urines examined, though as little as  $5 \times 10^{-8}$  gm. of this metal may be readily detected in 1 cc. of urine by the method employed. This absence of lead is particularly of interest, in view of the fact that, as our data clearly show

(Rabinowitch, '36) the Eskimo does develop arteriosclerosis. In the Eskimo, at least, therefore, some other cause must be sought for this disease.

Urines were also examined spectrographically for copper and, in general, the data agreed with the above-mentioned chemical findings.

According to the spectrograms, there were appreciable quantities of magnesium in some of the urines. The significance of these findings is not clearly understood. It is, however, of interest to note that, unlike all other flesh foods in the Eastern Arctic, seal meat has a purgative action and the Eskimo eats large quantities of seal.

Finally, of biological interest, and, as stated in the clinical report (Rabinowitch, '36) is the fact that no definite evidence of rickets was found in any of the 389 natives examined (there were three suspicious cases). The absence, or at least rarity, of this condition, especially at the latitudes of Clyde River ( $70^{\circ} 26'$ ), Pond Inlet ( $72^{\circ} 40'$ ), Dundas Harbour ( $74^{\circ} 35'$ ) and Craig Harbour ( $76^{\circ} 12'$ ) is instructive when consideration is given to the fact that the effectiveness of the sun's rays in the production of vitamin D-like substances decreases toward the Pole. Furthermore, the Eskimo's skin is quite pigmented. This should decrease penetration of the little of the sun's rays which are effective. The reason, however, for the absence of rickets is the practice of nursing infants until they are 2 and 3 or more years old and the fact that the vitamin D-content of seal oil is equal to that of the best cod liver oil.

#### SUMMARY

The average concentrations of non-protein nitrogenous constituents of the bloods of forty-six Eskimos were found to be higher than of peoples elsewhere and could not be explained by impairment of kidney function nor by conditions generally known to lead to such high values otherwise. Retention of nitrogen in the blood appears to be a physiological phenomenon among these Eskimos and is apparently due to the high protein diets.

The concentrations of chlorides in plasma were determined in thirty-four Eskimos and both maximum and average values were higher than the average renal threshold level of NaCl of peoples elsewhere. In spite of these high values, the concentrations of chlorides found in the urines were extremely low. The possible significance of these findings is briefly discussed.

None of the urines collected under ordinary conditions contained glucose or acetone according to the usual tests. The absence of glucose fits in with the absence of diabetes clinically (Rabinowitch, '36) and to explain the absence of ketonuria it does not appear necessary to seek for some unknown mechanism for utilization of fat. When food is plentiful, fat is not regarded as a relish and the carbohydrate contents of certain foods, the high protein intake and the potential sugar production from protein are such that the ratio of fatty acid to glucose is below the generally accepted level of ketogenesis.

Though there was no glycosuria and no clinical evidence of diabetes mellitus, the average blood sugar of thirteen Eskimos in the fasting state suggests some difficulty in the utilization of carbohydrates. The blood sugar time curves obtained in these individuals following ingestion of glucose clearly indicated that these Eskimos were unable to utilize large quantities of glucose in pure form.

Respiratory quotients lower than 0.7 were found in five of ten individuals in the fasting state and in three of eight tests in two individuals after administration of fat meals and, though conditions were not ideal for this type of work, no sources of error could be detected. The combined data, therefore, suggest that, though there is nothing peculiar about the absence of ketosis, it is still possible that the metabolism of fat in these Eskimos may to some extent be different from peoples elsewhere and that in these Eskimos may be found the solution to the controversy with respect to the conversion of fat to sugar.



The basal metabolic rates of these Eskimos appear to be high. The data fit in with the cold environment which is known to increase metabolism and also with the food habits of these people, in view of the high specific dynamic action of protein.

Urines were examined spectrographically with respect to a number of inorganic elements. No lead was found in any of the twenty-four specimens examined though the sensitivity of the method employed is approximately  $5 \times 10^{-8}$  gm. per cubic centimeter. The significance of these findings with respect to arteriosclerosis is briefly discussed.

The copper contents of the urines, as determined spectrographically, were essentially the same as found by chemical analysis.

According to the spectrograms, the urines contained relatively large quantities of magnesium. The explanation is not clear, but the large amounts of seal meat in the diets and the known purgative action of this meat are suggestive.

The absence or at least rarity of rickets among these Eskimos, in spite of their proximity to the Pole and pigmentations of the skin, appears to be due to the long periods during which infants are breast fed and the fact that the vitamin D-content of seal oil is equal to that of the best cod liver oil.

The above studies were part of a general investigation of the health of Eskimos in Canada's Eastern Arctic. Space does not permit mention of the many whose cooperation made this investigation possible. Due acknowledgment was, however, made in the report to the Dominion Government and in a previous communication (Rabinowitch, '36).

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# THE INFLUENCE OF VITAMINS A, B OR D, ANEMIA OR FASTING UPON THE RATE OF FAT ABSORPTION IN THE RAT <sup>1, 2</sup>

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## INTRODUCTION

In a preceding communication we reported differences in the rate of absorption of various fats (Steenbock, Irwin and Weber, '36). Of the fats tested halibut liver oil, cod liver oil and butter oil were absorbed the most rapidly. As these fats are distinguished from the others in containing considerable quantities of vitamin A and some vitamin D, it seemed desirable to determine if these vitamins were responsible for the differences.

That vitamins play a role in the absorption of foodstuffs has been suggested previously. For example, there have been published numerous reports to the effect that the secretory and motor activities of the digestive tract are decreased in avitaminosis (Boldyreff, '31; Hagashi, '30; Menville, Ane and Blackberg, '31; Menville, Blackberg and Ane, '29; Mott-ram, Cramer and Drew, '22; Murakami, '28; Never, '28, '30; Pierce, Osgood and Polansky, '29; Webster and Armour, '33; and Yonemura, '27). Gal ('30) and Kokas and Gal ('29) using technic similar to that used in our experiments have shown that the rate of absorption of protein and carbohydrate

<sup>1</sup> The authors wish to express their appreciation of the financial support of the Lever Brothers Company, which made this work possible.

<sup>2</sup> Published with the permission of the director of the Wisconsin Agricultural Experiment Station, Madison.

is decreased in B avitaminosis and that a yeast extract is capable of stimulating the absorption of sugar and of peptone. On the other hand, the total digestibility of a foodstuff has been stated not to be affected by a lack of any one of the vitamins (Bergeim, '28; St. Julian and Heller, '31; and Sampson, Dennison and Korenchevsky, '32). Because of the natural occurrence of vitamin A in fats a number of investigators have associated vitamin A with fat metabolism. Domagk and Dobeneck ('33) have suggested that vitamin A bears the same relationship to fat metabolism that vitamin D does to calcium metabolism. Our investigations, accordingly, concerned themselves with the effect of vitamins A, B and D on fat absorption. Incidentally, we also determined the effect of anemia and fasting.

#### EXPERIMENTAL

The rate of absorption of fat was determined by the method previously described by Irwin, Steenbock and Templin ('36), which is a modification of the technic used by Cori ('25) for determining the rate of absorption of sugar. Adult rats were fasted 48 hours, fed 1.5 cc. of melted fat by stomach tube, killed after a definite time interval, and the residual fat in the alimentary canal determined after 4 hours. A 4-hour absorption period was used because this period proved to be the critical one. Both male and female rats were used, as we have shown that the percentage of fat absorbed in a definite time period is the same for both sexes (Irwin, Steenbock and Templin, '36). The fats<sup>3</sup> used were halibut liver oil, a vegetable shortening (fat A), and a vegetable salad oil (fat W).

*Vitamin A and fat absorption.* To determine if the more rapid rate of absorption of halibut liver oil could be due to its vitamin A content, an absorption test was carried out with oil in which the vitamin A had been destroyed by aeration at 100°C. Aeration was discontinued when the antimony tri-

<sup>3</sup> The fats and oils used were those available commercially. The halibut liver oil was obtained from Parke, Davis & Company. Hydrogenated vegetable shortening A was prepared by hydrogenating cottonseed oil to an iodine number of approximately 70. The saponification number was 196.0. Fat W was a refined cottonseed salad oil.

chloride test was negative, which required 36 hours of treatment. Of this oil only 58% was absorbed in contrast with 70% for the controls (table 1). The mean difference of 12% was significant as it gave a standard deviation of 4.2 (table 2).

Further data were obtained by determining the effect of vitamin additions to the aerated oil. A vitamin A-concentrate was prepared in the following manner. Halibut liver oil was

TABLE 1

*The percentage of fat absorbed in 4 hours by rats fed 1.5 cc. of fat*

GROUP	FAT AND SUPPLEMENTS	CONDITION OF RATS	NUMBER OF RATS	RANGE IN WEIGHT	FAT ABSORBED
				<i>gm.</i>	<i>%</i>
1	Halibut liver oil	Normal	10	245-345	70 $\pm$ 2.0
2	Aerated halibut liver oil	Normal	6	225-315	58 $\pm$ 2.0
3	Aerated halibut liver oil + 28,000 blue units of vitamin A per cubic centimeter	Normal	10	240-338	54 $\pm$ 2.1
4	Fat A	Normal	11	236-392	54 $\pm$ 1.6
5	Fat A + 26,400 blue units of vitamin A per cubic centimeter	Normal	11	275-350	49 $\pm$ 1.1
6	Fat A	Avitaminotic A	11	98-134	30 $\pm$ 0.9
7	Fat A + 24,200 blue units of vitamin A per cubic centimeter	Avitaminotic A	4	107-140	31 $\pm$ 1.6
8	Fat A	Young, normal	5	125-145	56 $\pm$ 2.4
9	Fat W	Normal	10	275-382	56 $\pm$ 1.7
10	Fat W	Avitaminotic B	11	217-302	45 $\pm$ 1.6
11	Fat W	Avitaminotic D	12	100-160	37 $\pm$ 2.0
12	Fat A + yeast extract	Normal	5	250-300	49 $\pm$ 2.4
13	Fat A + 320 International units of vitamin D per cubic centimeter	Normal	10	280-426	58 $\pm$ 1.3
14	Fat A	Anemic	4	90-124	45 $\pm$ 1.6
15	Fat A	Reared on limited amount of stock ration	8	103-150	35 $\pm$ 1.0
16	Fat A	Fasted 6 days	10	290-365	44 $\pm$ 1.6

saponified, extracted with ether, and the ether distilled off in a partial vacuum with nitrogen. The residue was taken up in methyl alcohol and allowed to stand for 12 hours at 1°C. The sterols which crystallized out were filtered off. The methyl alcohol was dissipated in a partial vacuum under nitrogen and the residue dissolved in aerated halibut liver oil to give a concentration of 28,000 blue units per cubic centimeter. Of this mixture the rats absorbed 54 %—only 4% less than the control with no vitamin A addition, which is not statistically significant. Apparently then, in the preceding experiment the

TABLE 2  
*Significance of the differences in absorption*

DIFFERENCE BETWEEN	MEAN DIFFERENCE	STANDARD DEVIATION OF MEAN DIFFERENCE	$\frac{MD}{\sigma MD}$
	%		
Groups (1) and (2)	12	4.2	2.9
Groups (2) and (3)	4	4.3	0.9
Groups (4) and (5)	5	2.9	1.7
Groups (4) and (6)	24	2.7	8.9
Groups (9) and (10)	11	3.5	3.1
Groups (9) and (11)	19	3.9	4.9
Groups (4) and (12)	5	4.3	1.2
Groups (4) and (13)	4	3.0	1.3
Groups (4) and (14)	9	3.3	2.7
Groups (4) and (15)	20	2.7	7.4
Groups (4) and (16)	10	3.3	3.0

lowered rate of absorption of the aerated oil was not due to the destruction of vitamin A per se, but probably was caused by changes in the character of the glycerides induced by oxidation. As other methods of vitamin A removal than destruction by oxidation were not available, we were forced to limit further trials to determinations of the effect of vitamin A when added to an unoxidized fat. Such experiments were carried out with our halibut liver oil concentrate added to a hydrogenated fat (fat A) to a concentration of 26,400 blue units per cubic centimeters. This mixture was absorbed to the extent of 49%. Of the control fat without vitamin A, 54% was absorbed.

The effect of vitamin A-depletion in the animal upon the rate of fat absorption was also determined. Young rats weighing from 60 to 80 gm. were placed on a vitamin A-free diet for 7 weeks. They then weighed from 98 to 134 gm. (average 118 gm.) and showed signs of xerophthalmia. They were fasted 48 hours, then fed 1.5 cc. of fat A. Only 30% of the fat administered was absorbed. Later a group of four vitamin A-deficient rats weighing 107 to 140 gm. (average 124 gm.) was fed the same fat carrying 26,400 blue units of vitamin A per cubic centimeter. The mean percentage of absorption was essentially the same, namely 31%. However, since the vitamin A-deficient rats were smaller than the adult rats previously used as controls, additional control tests were carried out with smaller animals. The rats used for these tests weighed from 125 to 140 gm. (average 133 gm.). They were found to absorb 56% of the fat in comparison with 54% for the adults. It was, therefore, evident that the size of the animals per se was not responsible for the lower values obtained with the vitamin A-deficient rats.

*Effect of avitaminosis B.* A group of adult rats was fed a vitamin B-deficient diet (Kemmerer and Steenbock, '33) for 5 weeks. At the end of this time they showed symptoms of polyneuritis and had lost on an average 58 gm. in weight. Their weights ranged from 198 to 284 gm. After fasting for 48 hours they were given 1.5 cc. of fat W. Forty-five per cent of the fat was absorbed in 4 hours, whereas the controls, normal adult rats, absorbed 56%. The mean difference (11%) justifies the conclusion that vitamin B-deficient rats do not absorb fat as rapidly as normal animals.

To determine if a yeast extract would stimulate the absorption of fat, five normal rats were fed fat A as before, but 1 cc. of yeast extract was given directly after the fat. The yeast extract was prepared by treating 500 gm. of yeast containing 20 International units of vitamin B per gram with 1500 cc. of 70% alcohol containing 0.5% hydrochloric acid. The mixture was heated to boiling, allowed to stand 2 hours, filtered, and the filtrate concentrated in a partial vacuum at 50°C. The

concentrate was extracted three times with ether to remove the lipoids. Any ether remaining in the concentrate was removed by evaporating in a partial vacuum at 50°C. Rats given 1 cc. of this solution, equivalent to 3.3 gm. of yeast, absorbed 49% of the fat fed, whereas the controls absorbed 54%. The addition of the yeast extract, therefore, did not affect the percentage of absorption over that of the control group (table 2).

*Effect of vitamin D.* This was determined with young rats weighing approximately 135 gm. (range 100 to 160) which had been fed the Steenbock and Black ('25) rachitogenic diet no. 2965 for 7 weeks when they began to lose weight and had developed definite signs of rickets. Serum from a pooled blood sample from six of these rats contained 4.0 mg. of inorganic phosphorus per 100 cc. The skeletons revealed wide metaphyses and enlarged costochondral junctions. Six rats from this group absorbed fat W to the extent of 37%, whereas normal animals absorbed 56%.

We also determined if vitamin D added to fats would increase the rate of fat absorption in normal rats. To 1.5 cc. of a sample of fat A there was added a sufficient quantity of a standardized preparation of irradiated ergosterol to give a concentration of 320 International units of vitamin D per cubic centimeter. Fifty-eight per cent of this fat was absorbed in 4 hours. Of the control fat 54% was absorbed.

*Effect of anemia.* Since all of the animals on vitamin-low diets failed to absorb fat so readily as animals on adequate diets and since such animals were generally in a poor physical condition, the absorption of fat was studied in rats in a similar poor physical condition as induced by other means. Among these was the effect of anemia.

Four anemic rats 11 weeks of age, weighing 90 to 124 gm. (average 107 gm.), that had been reared on a milk diet and had 2.4, 3.6, 3.6 and 4.1 gm. of hemoglobin per 100 cc. of blood, respectively, were fed 1.5 cc. of fat A. These animals absorbed 45% of the fat fed. This is significantly less than the amount absorbed by normal animals.



*Effect of starvation.* Another factor studied was starvation. A group of young rats weighing 60 to 80 gm. was fed a limited quantity of a stock diet (Steenbock, '23) for a period of 6 weeks. At the end of this time they weighed from 103 to 150 gm. This is an abnormally low weight because similar animals fed ad libitum would have weighed from 200 to 300 gm. at this age. These semi-starved animals absorbed 35% of the fat fed, whereas normal rats absorbed 54%.

In a second experiment adult male rats weighing 290 to 365 gm. were fasted for 6 days and then fed 1.5 cc. of fat. Forty-four per cent of the fat was absorbed in 4 hours, which is 10% less than that absorbed by the controls. It is evident that a decreased rate of absorption in avitaminosis may be ascribed secondarily to a poor physical condition rather than to the specific lack of a vitamin or vitamins.

#### SUMMARY

1. Avitaminotic A, B or D animals absorb fat less rapidly than normal animals, which effect is not specific for any one of the vitamins tested.

2. The addition of vitamins A, B or D to a fat had no effect upon its rate of absorption by normal rats.

3. Anemic animals, those fasted 6 days, or those fed a limited quantity of a complete diet also absorbed fat less rapidly than controls.

4. As the experiments on anemia and semi-starvation showed that the rate of fat absorption was influenced by the nutritional state of the animal, it cannot be concluded that the vitamin deficiency experiments revealed any specific effects of vitamins on fat absorption.

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# THE INFLUENCE OF CERTAIN HYDROTROPIC AND OTHER SUBSTANCES UPON FAT ABSORPTION<sup>1, 2</sup>

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## INTRODUCTION

Neuberg ('16 a, b) has shown that certain hydrotropic substances are capable of rendering soluble and diffusible, compounds, which of themselves are insoluble in water. The work of Verzar and Kuthy ('29 a, b, c, '30, '31), Verzar ('31), Wieland and Reverey ('24), Wieland and Sorge ('16) and Reinboldt ('27) has shown that bile acids combine with fatty acids, forming complex aggregations which are diffusible and stable within the reaction range prevailing in the intestine. It is quite possible that the rate of absorption of a foodstuff may depend not only on the functional activity of the digestive organs, but on the presence of hydrotropic substances in the food mixture. In this laboratory a variety of substances have been fed with fats in order to determine the effect of these substances upon the rate of fat absorption.

## EXPERIMENTAL

Adult rats that had been fasted 48 hours were lightly anesthetized with ether and fed 1.5 cc. of melted fat by stomach tube. Immediately following the fat feeding 1 cc. of a water solution of the substance under investigation was fed, also by stomach tube. After a 4-hour absorption period the rat was killed and the amount of fat which had been absorbed was determined by analyzing the residues in the intestinal tract.

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<sup>2</sup> Published with the permission of the director of the Wisconsin Agricultural Experiment Station, Madison.

TABLE 1

*The percentage of fat absorbed in 4 hours by rats fed 1.5 gm. of fat followed by 1 cc. of a solution containing the substance being tested*

GROUP	TREATMENT	NUMBER OF ANIMALS	PERCENTAGE OF FAT ABSORBED
	Controls:		
1	Fed 1.5 cc. fat only	11	54 $\pm$ 1.6
2	Fed 1.5 cc. fat plus 1 cc. distilled water	10	55 $\pm$ 1.9
	Effect of bile. Fed 1.5 cc. of fat plus 1 cc. of a solution containing:		
3	1 mg. commercial bile salts	5	53 $\pm$ 2.4
4	50 mg. commercial bile salts	5	55 $\pm$ 1.9
5	500 mg. commercial bile salts	6	19 $\pm$ 2.3
	Effect of sodium benzoate. Fed 1.5 cc. of fat plus 1 cc. of a solution containing:		
6	1 mg. sodium benzoate	6	50 $\pm$ 2.0
7	50 mg. sodium benzoate	6	38 $\pm$ 2.5
8	500 mg. sodium benzoate	3	14 $\pm$ 3.3
	Effect of ethyl alcohol. Fed 1.5 cc. fat plus 1 cc. of a solution containing:		
9	5% alcohol	7	51 $\pm$ 1.7
10	15% alcohol	6	47 $\pm$ 1.8
11	40% alcohol	8	27 $\pm$ 5.8
	Effect of peptone, sucrose and a mixture of peptone and sucrose. Fed 1.5 cc. of fat plus 2 cc. of a solution containing:		
12	1 gm. peptone	5	27 $\pm$ 1.5
13	1 gm. sucrose	5	33 $\pm$ 2.4
14	1 gm. peptone and 1 gm. sucrose	6	25 $\pm$ 2.6
	Effect of KCl. Fed 1.5 cc. of fat plus 1 cc. of a solution containing:		
15	0.2 mg. potassium chloride	10	48 $\pm$ 1.5
16	20.0 mg. potassium chloride	10	55 $\pm$ 1.2
17	100.0 mg. potassium chloride	7	34 $\pm$ 2.6
	Effect of CaCl <sub>2</sub> . Fed 1.5 cc. of fat plus 1 cc. of a solution containing:		
18	0.2 mg. calcium chloride	6	54 $\pm$ 2.5
19	20.0 mg. calcium chloride	6	54 $\pm$ 3.2
20	100.0 mg. calcium chloride	6	26 $\pm$ 2.9
	Effect of NaH <sub>2</sub> PO <sub>4</sub> and glycerol. Fed 1.5 cc. of fat plus 1 cc. of a solution containing:		
21	2 mg. NaH <sub>2</sub> PO <sub>4</sub>	6	47 $\pm$ 1.2
22	10% glycerol	7	43 $\pm$ 2.5
	Effect of sodium glycerophosphate. Fed 1.5 cc. of fat plus 1 cc. of a solution containing:		
23	2 mg. sodium glycerophosphate	7	47 $\pm$ 1.3
24	50 mg. sodium glycerophosphate	7	38 $\pm$ 2.0
25	500 mg. sodium glycerophosphate	7	29 $\pm$ 2.7

The animals were male and female rats from our stock colony and were 4 to 6 months of age. We have shown previously that there is no significance difference between the percentages of fat absorbed by male and female animals. For details of the method used and for tests dealing with the accuracy of the technic, etc., the reader is referred to the first paper of this series (Irwin, Steenbock and Templin, '36).

TABLE 2  
*Significance of differences in absorption*

DIFFERENCE BETWEEN	MEAN DIFFERENCE	STANDARD DEVIATION OF MEAN DIFFERENCE	$\frac{MD}{\sigma MD}$
	%		
Groups (1) and (2)	1	3.8	0.3
Groups (1) and (5)	35	4.1	8.5
Groups (1) and (6)	4	4.0	1.0
Groups (1) and (7)	16	4.4	3.6
Groups (1) and (8)	40	5.5	7.3
Groups (1) and (9)	3	3.5	0.9
Groups (1) and (10)	7	3.6	1.9
Groups (1) and (11)	27	8.8	3.1
Groups (1) and (12)	27	3.3	8.2
Groups (1) and (13)	21	4.2	5.0
Groups (1) and (14)	29	4.6	6.3
Groups (1) and (15)	6	3.3	1.8
Groups (1) and (16)	1	3.0	0.3
Groups (1) and (17)	20	4.5	4.4
Groups (1) and (20)	28	4.8	5.8
Groups (1) and (21)	7	3.0	2.3
Groups (1) and (22)	11	4.2	2.6
Groups (1) and (23)	7	3.0	2.3
Groups (1) and (24)	16	3.7	4.3
Groups (1) and (25)	25	4.4	5.7

The base fat fed was a commercially hydrogenated vegetable shortening (fat A)<sup>3</sup> which was chosen because of its uniform composition and stability. Data showing the percentages of fat absorbed in 4 hours by adult rats fed 1.5 cc. of the fat followed by 1 cc. of a solution of the various substances tested are given in table 1.

Table 2 records the statistical significance of the differences in the means of the several groups.

<sup>3</sup>Fat A was a commercial product manufactured by hydrogenating cottonseed oil to an iodine number of approximately 70. The saponification number was 196.0.

## DISCUSSION

*Bile salts.* Verzar and Kuthy ('29 b) have advanced the theory that the bile acids aid fat absorption by combining with the fatty acids, 'carrying' them through the intestinal wall. In a more recent publication Verzar and Laszt ('34) report an experiment in which they anesthetized rats with urethane, ligated a loop of the intestine—leaving the circulation intact, and after washing out the loop with physiological saline, injected into it a mixture of oleic acid and bile acid. After a definite time interval the amount of fatty acid absorbed was determined. They found that the absorption of oleic acid was definitely increased by glycocholic acid under the conditions of their experiment. In our experiments the addition of 1 or 50 mg. of commercial bile salts<sup>4</sup> did not increase the rate of absorption of fats. The addition of 500 mg. decreased absorption, although this figure (19%, table 1) is not a reliable one due to the laxative effect of this quantity of bile salts. No indications of diarrhea were observed in our animals when 1 or 50 mg. of bile salts were fed. In Verzar's experiment normal secretions into the intestinal tract were in part excluded; in ours, the digestive tract remained intact during the absorption period with the secretions and movements of the intestine functioning normally. Possibly in our experiments the optimum amount of bile acids was secreted by the liver, and, therefore, the further addition of small amounts of bile salts had no effect.

*Sodium benzoate.* Sodium benzoate is one of the many organic salts which shows hydrotropic properties. When fed with fat, 1 mg. did not affect the rate of absorption, but 50 or 500 mg. definitely lowered it (table 1).

*Alcohol.* Cori, Villiaume and Cori ('30) observed that small quantities of ethyl alcohol did not alter the rate of absorption of sugar. We obtained similar results with fat; a small quantity of alcohol had no marked effect on fat absorption, although 1 cc. of a 40% alcohol solution decreased it from 54% to 27% (table 1).

<sup>4</sup>Supplied through the kindness of the Wilson Laboratories. The preparation consisted predominately of crude sodium glycocholate and sodium taurocholate.

*Peptone and sucrose.* Since, in the ordinary diet, fat is usually absorbed in the presence of protein and carbohydrate, fat was fed with peptone, with sucrose, and with a mixture of the two. From the data presented in table 1 it is clear that fat was absorbed less rapidly when fed with these foodstuffs than when fed alone.

*Calcium and potassium.* The effect of electrolytes on absorption is, as yet, an unsettled question. Magee and Reid ('27), Magee and Southgate ('29), and Magee and Sen ('31, '32) have observed that small amounts of  $\text{CaCl}_2$  in the lumen of the rabbit's intestine increased its tone and stimulated contraction. Glucose diffused more rapidly from sections of the surviving intestine into a Tyrode solution containing calcium than it did into a calcium-free solution. Magee and co-workers found also that thyroparathyroidectomized rats with a low blood calcium did not absorb as much glucose as normal rats and that the addition of calcium to the sugar solution increased absorption.

Contrary evidence has been presented by Gellhorn and Skupa ('33) who perfused the gut of frogs with a glucose solution and the blood vessels with a modified Ringer's solution. By the addition or omission of one salt or another these investigators found that when both calcium and potassium were omitted from the perfusate, permeability of the gut to glucose was increased. The addition of  $\text{CaCl}_2$  alone to such a solution decreased permeability, but when as little as 0.0075% of KCl was added, the permeability of the gut to glucose was increased.

Whether or not the action of calcium and potassium ions is specific for glucose absorption or is fundamental to the process as a whole cannot be determined until more evidence is presented. Under the conditions of our experiment small quantities of  $\text{CaCl}_2$  or KCl had no effect on fat absorption; larger amounts of either of these salts depressed absorption (table 1).

*Phosphates.* Verzar and Laszt ('34) obtained a definite increase in the absorption of oleic acid when glycerophosphate was injected into ligated loops of rat's intestine along with oleic acid and bile acids. Sinclair ('29), Verzar and Laszt ('34),

and also Süllmann and Wilbrandt ('34) believe that the transformation of absorbed fatty acids into phospholipids is an essential step in the resynthesis of neutral fat in the intestinal mucosa. Additional evidence supporting this idea is to be found in the work of Smedley MacLean and Hoffert ('23) who found that yeast grown in an oxygenated medium containing phosphates stored much more fat than when grown on a phosphate-free medium. Magee and Reid ('31) reported that the presence of 0.2%  $\text{NaH}_2\text{PO}_4$  in a 0.75 M glucose solution increased its rate of absorption by 50%. This work has been confirmed by Wilbrandt and Laszt ('33), and it has been suggested that the formation of glycerophosphoric acid is a step in the absorption of glucose. In our experiments  $\text{NaH}_2\text{PO}_4$ , glycerol, and sodium glycerophosphate solutions were fed along with fat, and the absorption of the fat determined. In table 1 it can be seen that the presence of any one of these substances in the intestinal tract during digestion and absorption of fat retarded rather than stimulated the process.

#### SUMMARY

The influence of water, bile salts, sodium benzoate, ethyl alcohol, peptone, sucrose,  $\text{KCl}$ ,  $\text{CaCl}_2$ ,  $\text{NaH}_2\text{PO}_4$ , glycerol and sodium glycerophosphate on the rate of absorption of fats has been determined. In general the feeding of small amounts of any one of these substances had little or no effect, but larger amounts invariably decreased the rate of fat absorption.

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# RELATION OF CALCIUM AND OF IRON TO THE ERYTHROCYTE AND HEMOGLOBIN CONTENT OF THE BLOOD OF RATS CONSUMING A MINERAL DEFICIENT RATION<sup>1</sup>

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TWO FIGURES

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The fact that extreme restriction of the inorganic constituents of the diet produces significant alterations in the concentration of pigment and of erythrocytes in the blood of the rat has been repeatedly demonstrated (Smith and Schultz, '30; Swanson and Smith, '32; Orten and Smith, '34 a). There occurs a progressive increase in the number of erythrocytes per unit volume of blood and a simultaneous decrease in the concentration of hemoglobin. Careful analyses of the diet employed (Smith and Smith, '34) have shown that it is extremely deficient in calcium and, to a lesser extent, in potassium, sodium, phosphorus, magnesium, and chloride. Likewise, the amount of iron present in the low-salt ration (Brooke, '33) is somewhat less than that commonly believed to be required by the normal albino rat.

There arises the question of the nature of the specific inorganic deficiency or deficiencies responsible for the polycythemia and concomitant anemia observed in the low-salt animals. Preliminary studies demonstrated that the blood

<sup>1</sup> A preliminary report was made before the meeting of the Society of Biological Chemists in New York, March, 1934. Aided by a grant-in-aid from the National Research Council, 1934.

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<sup>3</sup> Deceased.

changes were due solely to a lack of inorganic elements since the administration of the complete Osborne and Mendel salt mixture to the polycythemic rats restored the levels of pigment and of erythrocytes to normal. In view of the well-known relation of iron to hemoglobin formation, an involvement of this metal in the low-salt hematological syndrome was early suspected, even though it was realized that an iron deficiency usually produces a decrease in the erythrocyte content of the blood as well as a decrease in the concentration of pigment. The extreme deficiency of calcium in the experimental ration and the suggested relation (von Wendt, '05; Sherman, '07) of this element to the economy of the metabolism of iron also indicated a possible relation of calcium to the observed hematological abnormalities. For these reasons, the effect of the administration of iron and of calcium on the blood of rats consuming a mineral deficient ration was investigated.

#### EXPERIMENTAL

Male albino rats of the Connecticut Agricultural Experiment Station strain, weighing from 40 to 50 gm. at weaning (21 days of age), were employed as experimental animals. They were housed in individual wire cages having a wide mesh ( $\frac{1}{2}$  inch) bottom designed to minimize the opportunity for coprophagy. The weanling animals were fed the stock colony ration (Orten and Smith, '34 b) until they attained a weight of  $120 \pm 4$  gm. In order to further insure uniform, vigorous experimental subjects, only those animals which attained the above weight at  $35 \pm 2$  days of age were used. These rats were then subjected to two general types of experimental procedures, the 'curative' and the 'preventive,' described below.

*Curative procedure.* For a preliminary period of 8 weeks, the 35-day-old animals were fed a basal 'low-salt' ration having the following composition: Specially prepared low-ash casein, 18%; dextrin, 55%; hydrogenated vegetable oil,<sup>4</sup>

<sup>4</sup> Crisco.

27%. The daily vitamin supplements consisted of wheat germ extract, 1 cc.; dried yeast, 200 mg.; cod liver oil, 100 mg. and wheat germ oil, 40 mg. The source of the various dietary constituents and the methods of preparation have been described elsewhere (Swanson and Smith, '32; Swanson, '30). At the end of the 8-week preliminary period, the various mineral supplements to be studied were added to the ration and the animals were observed for a further 10-week experimental period. Calcium was fed as 'C.P.' calcium carbonate at a level of 50 mg. calcium daily, the amount ingested daily in the form of the salt mixture (Osborne and Mendel, '17) by comparable normal control animals. This quantity of calcium should be sufficient to satisfy the daily requirement of the adult rat (Osborne and Mendel, unpublished data; Whitcher, Booher and Sherman, '33), even during pregnancy and lactation (Cox and Imboden, '36). Iron was fed as purified, copper-free ferric chloride<sup>5</sup> at a level of 1.4 mg. daily, the amount consumed daily by the normal control rats. Three groups of control animals were studied. One of these was continued on the basal low-salt ration; another was given the complete salt mixture as a supplement, at a level of 408 mg. daily, the average amount ingested by normal control animals; and the third group was given chloride at a level of 2.34 mg. HCl daily (N/10 solution), as a control to the ferric chloride-supplemented group. In all cases, the mineral supplements were mixed directly in the basal ration which was fed daily in an amount equal to the average daily quantity consumed by the animal during the final 2 weeks of the preliminary period. The reason for the feeding of this 'restricted' allowance of the basal ration was to prevent possible changes in the amount of basal ration consumed after supplementation, a variation which would have rendered difficult an interpretation of subsequent changes in the composition of the blood in terms of the inorganic supplement per se. Normal control animals

<sup>5</sup> Appreciation is expressed to Dr. W. W. Watson of the Department of Physics for a spectrographic examination of this sample of ferric chloride.

were maintained during the entire preliminary and experimental periods. These received an ad libitum allowance of the basal low-salt ration, in which 4% salt mixture replaced an equal amount of dextrin, and the same amounts of vitamin supplements as the experimental animals.

*Preventive procedure.* The young rats, 35 days old, were changed directly from the stock colony ration to the mineral-supplemented low-salt ration and observed for a 10-week period. Calcium, as the 'C.P.' or specially purified, iron-free carbonate, and iron, as the purified chloride or the citrate, were fed in the same daily amounts described above. Control animals were fed either the unsupplemented low-salt ration, or the ration supplemented with hydrochloric acid, or with the entire salt mixture. In all instances, the inorganic adjuvants were incorporated directly in the basal ration. In order to keep the mineral supplement as the sole controllable variable in the experiment, all members of the various groups received the same 'restricted' amount of food daily. This quantity was calculated from previous food consumption records<sup>6</sup> of 105 rats receiving the same basal diet as that employed in the present work. The daily allowance of the basal ration, adjusted weekly to that of the recorded averages, was as follows: First week, 7.1 gm.; second week, 6.4 gm.; third to fifth weeks, 5.7 gm.; sixth to tenth weeks, 5.6 gm.

The body weights of the various animals were recorded weekly and the hemoglobin content and erythrocyte count of the blood were determined bi-weekly during the entire period of observation. The methods employed for the blood determinations have been described elsewhere (Orten and Smith, '34 b).

## RESULTS

The averaged data obtained in this investigation, with the exception of the body weight values, are given as composite curves in the accompanying charts. Some of the body

<sup>6</sup>Appreciation is expressed to Dr. Miriam F. Clarke and Dr. Paul K. Smith for making available the food intake data collected on their rats fed the low-salt ration.

weight findings may be described briefly. The normal control animals used in the 'curative' study grew at a rate similar to that of comparable stock rats (Orten and Smith, '34 b) while those consuming the low-salt ration showed a typical retardation of growth (Swanson and Smith, '32), reaching and maintaining an average maximum body weight of approximately 160 gm. The animals given the supplement of the complete salt mixture at the end of the preliminary 8-week period increased on the average approximately 40 gm. in weight during the 10-week realimentation period while those given only calcium gained some 20 gm. The animals receiving ferric chloride, however, progressively lost weight during the entire realimentation period. Similar results were obtained in the animals studied by the 'preventive' procedure. At the end of the 10-week experimental period, the groups given either the complete salt mixture or calcium had increased approximately 20 gm. more in body weight than the low-salt controls, whereas those given ferric chloride (or HCl) weighed slightly less than the unsupplemented controls. It should be borne in mind that the growth effects described are due solely to the influence of the various inorganic supplements since the allowance of energy-yielding basal ration in all cases was 'restricted,' and therefore did not vary between groups.

The averaged erythrocyte and hemoglobin data obtained on the groups of rats studied by the 'curative' procedure are given in figure 1. The erythrocyte counts and pigment concentrations of the blood of the normal control animals fed the adequate ration showed the characteristic increase with age (Orten and Smith, '34 b) and attained adult levels after some 6 weeks on the experiment. The animals fed the low-salt ration, however, developed the typical polycythemia and mild anemia. These abnormal values persisted in the animals continued on the low-salt ration, although there was a tendency for the average erythrocyte count of the group to decrease because of a marked premortal fall in the value in certain members which succumbed during the experiment. The addition of the complete salt mixture to the low-salt diet at the

end of the 8-week preliminary period was followed by a prompt, progressive decrease in the erythrocyte count and an increase in pigment concentration to normal values. Similar

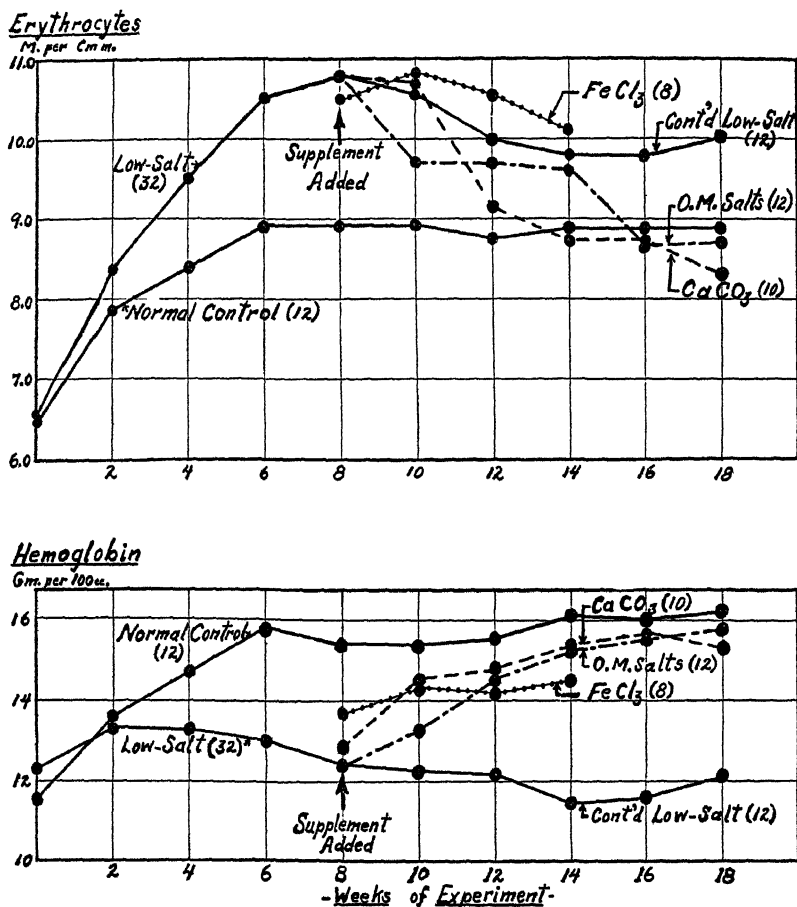


Fig.1 'Curative procedure.' Average erythrocyte and hemoglobin content of the blood of control rats and of rats consuming a 'low-salt' ration supplemented by certain inorganic elements. The figures in parentheses indicate the numbers of rats in the groups.

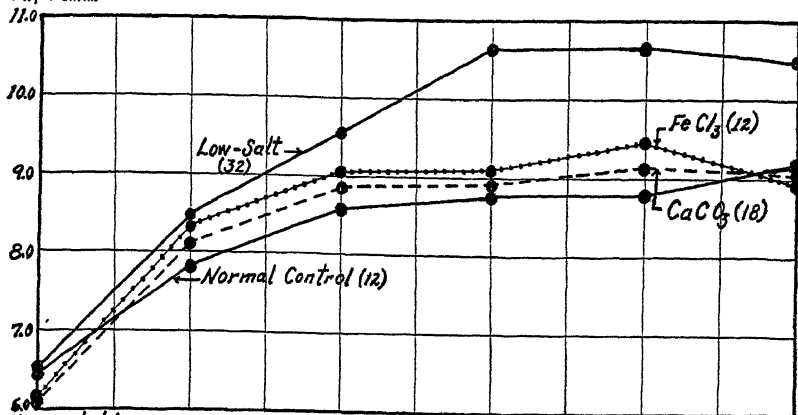
results were obtained with calcium; without exception, there was a restoration of the cell count and hemoglobin level to values within the normal range. There was a similar tendency in some of the animals fed ferric chloride. However,



the results were not convincing because the responses to iron were exceedingly variable, some animals showing a decrease in erythrocytes and a rise in hemoglobin, others showing

### Erythrocytes

M. per Cmm.



### Hemoglobin

Gm. per 100cc.

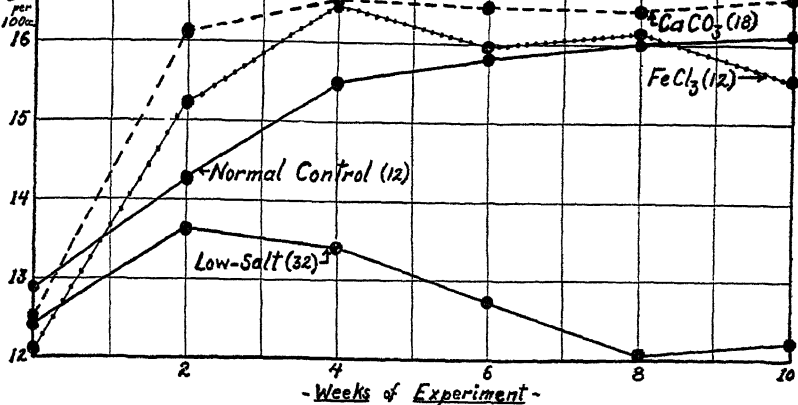


Fig. 2 'Preventive procedure.' Average erythrocyte and hemoglobin content of the blood of control rats and of rats consuming a 'low-salt' ration supplemented by certain inorganic elements. The figures in parentheses on the various curves indicate the numbers of rats in the groups.

either no change or a slight aggravation of the condition, and because the majority of the animals died after a few weeks, some within a few days, of supplementation. The findings in animals fed hydrochloric acid (not included in the chart) did

not differ significantly from those in the animals continued on the basal ration alone.

The averaged blood data for the groups of rats studied by the 'preventive' procedure are given in figure 2. The animals given the basal low-salt ration to which the complete salt mixture had been added maintained normal erythrocyte counts and hemoglobin values during the entire experimental period, whereas those given the unsupplemented low-salt ration developed the usual polycythemia and concomitant mild anemia. In all cases the rats given calcium alone as a supplement showed erythrocyte counts and pigment concentrations differing but little at any time during the entire experiment from those of the animals receiving the complete salt mixture. Similar results were obtained in the group fed ferric chloride, although there was a distinct tendency toward the end of the 10-week period for the concentration of hemoglobin in these animals to fall below that of the rats given either the entire salt mixture or calcium alone. The findings on the 'chloride control' group (not given in the figure) differed little from those of the animals fed the unsupplemented low-salt ration. The response of the group of rats fed ferric citrate (not included in the chart) was similar to that of the rats given ferric chloride with the exception that the hemoglobin level was consistently lower. At the end of the experiment, the average hemoglobin concentration of the blood of these animals was only 14.0 gm. per 100 cc. blood, in contrast to the value, 15.4 gm., obtained on the group fed ferric chloride.

#### DISCUSSION

From the results obtained with the groups of animals receiving the complete salt mixture, it is evident that the polycythemia and concurrent mild anemia observed in rats fed a diet deficient in inorganic salts are due exclusively to the low mineral intake. Furthermore, the fact that purified calcium alone produces a similar effect in the restoration or maintenance of normal erythrocyte and hemoglobin values indicates that a lack of this element is intimately involved in the

hematological abnormalities observed. Likewise, the response obtained with iron in the 'preventive' study points to an involvement of this element. Additional experiments (unpublished observations) have indicated that the other common dietary inorganic elements, sodium, potassium, magnesium, and chloride, are not concerned in this condition, since neither the adding of these elements to the basal low-salt ration nor the feeding of a salt mixture devoid of them produces any alteration in the results otherwise obtained. However, the fact that the substitution of edestin, a protein containing little, if any, phosphorus, for the phosphoprotein, casein, results in less drastic changes in the composition of the blood (Swanson, Timson and Frazier, '35) suggests that phosphorus, or perhaps the calcium to phosphorus ratio, may be involved in this condition. The foregoing results warrant the conclusion that the hematological abnormalities occurring in rats fed the diet deficient in inorganic salts are due largely, if not entirely, to the lack of calcium and/or iron.

There are certain suggestions in the literature which may serve as a basis for an explanation of the favorable effects obtained with iron and with calcium in this study. It has been observed (Eveleth, Bing and Myers, '33) that rats fed a diet of whole cow's milk, apparently containing a sufficient amount of copper, supplemented by a minimal amount of iron did not show the usual iron-deficiency anemia but developed a strikingly polycythemia accompanied by a subnormal level of hemoglobin. The administration of larger doses of iron, on the other hand, produced normal levels of erythrocytes and hemoglobin in the blood, thus demonstrating that the polycythemia-anemia was due entirely to a lack of a sufficient amount of iron. This hematological syndrome and its relation to iron is remarkably similar to that observed in the animals consuming the low-salt ration. It is possible that in both instances the polycythemia is merely a compensatory response, that is, a production of increased numbers of smaller-sized erythrocytes to compensate for a subnormal level of pigment due to the availability of only a minimal amount of

iron. If this is true in the case of the animals fed the mineral deficient ration, the beneficial action of iron may simply be a correction of a slight iron deficiency, thus promoting the maintenance of a normal concentration of hemoglobin in the blood and, in turn, a normal number of erythrocytes.

The failure of iron to exert a consistently favorable effect when administered as a curative agent merits some comment. The fact that a definite rise in the level of hemoglobin and a simultaneous decrease in the erythrocyte count occurred in some animals whereas little or no change or even a decrease in the concentration of pigment was observed in others may be interpreted as indicating that the response to iron was dependent upon the presence of some other substance. In view of the invariably favorable responses to calcium supplementation, it appears likely that the other substance is calcium itself and that the inconsistent results obtained with iron in the curative procedure may have been due to variations in the amounts of reserve calcium in the different animals. Likewise, the favorable effect of iron in the preventive study may also have been dependent upon the presence of reserve calcium in the young animals before depletion on the salt-poor, basal ration. Even in these animals, however, there was a distinct tendency toward the end of the 10-week period for the concentration of hemoglobin to decrease below that in the blood of rats given either the complete salt mixture or calcium alone.

The consistently favorable results obtained with calcium are perhaps more difficult to explain. Although there is some evidence which appears to indicate the contrary (Shelling and Josephs, '34; Kletzien, '35), it is possible that under certain conditions this element exerts a beneficial effect on the economy of iron in metabolism, as was suggested by the results of a limited number of balance studies in human subjects (von Wendt, '05; Sherman, '07). The recent finding (Ramage, Sheldon and Sheldon, '33) that there is a remarkable degree of correlation between the amount of calcium and that of iron present in the livers of human infants may be interpreted as further evidence in support of this view.

It is possible that under the conditions of the present experiment calcium added to the diet increases the absorption or utilization of iron and therefore that the favorable results obtained with this element are simply an indirect iron effect. Some support to the suggestion of an impairment of iron metabolism in the low-salt animals is given by the fact that a favorable hemoglobin response was not consistently elicited by iron, particularly when fed as the citrate, and even by ferric chloride toward the end of the preventive experiment. In this connection, the statement of one investigator (Davidson et al., '33) regarding the relation between calcium, iron, and anemia is of interest: ". . . a normal blood level can be maintained on a diet rich in calcium and moderate in iron but that an anemia will occur on the same iron intake if the calcium is reduced." Thus, the possibility that calcium exerts an 'iron-sparing action' under the conditions of the present experiment, must be considered in interpreting the results herein presented.

A number of instances are found in the clinical literature which also indicate a possible relationship between calcium, or some factor controlling its metabolism, and the amount of hemoglobin in the blood. For example, in proved cases of hyperparathyroidism, there is often an anemia (Albright, Aub and Bauer, '34) due, according to the authors, to the replacement of some of the marrow cavity with fibrous tissue. An explanation on a chemical basis would seem to be equally tenable. Likewise, in a case of acromegaly with osteoporosis (Scriver and Bryan, '35) an anemia was recorded. Other similar examples are found scattered widely through the clinical literature.

Thus, there is a considerable amount of evidence both from studies on experimental animals and on man indicating a possible relationship between calcium, or some factor controlling its metabolism, iron, and the concentration of hemoglobin in the blood. The data obtained in the present study appear to support such a view since, under the conditions employed, rats receiving the mineral deficient ration develop a

chronic anemia with an accompanying microcytic polycythemia whereas other animals consuming the same amount of the same diet under identical experimental conditions but given calcium maintain a normal concentration of pigment and erythrocytes in the blood.

#### SUMMARY

The administration of a complete salt mixture to rats fed a basal ration extremely low in inorganic salts both alleviates and prevents the marked polycythemia and concurrent mild anemia which otherwise persist or develop.

Calcium alone, fed as the 'C.P.' or highly purified carbonate, exerts a comparable restorative and preventive action.

Iron alone, fed as purified ferric chloride, shows a similar preventive effect but is not consistently efficacious as a restorative agent.

Further available data indicate that none of the other common inorganic elements known to be deficient in the salt-poor diet, with the possible exception of phosphorus, are concerned in the production of the blood changes under discussion.

It appears, therefore, that the hematological abnormalities which occur in rats as a result of the feeding of the mineral deficient ration are due chiefly, if not entirely, to a lack of calcium and/or iron.

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# THE INVERSE RELATION BETWEEN GROWTH AND INCIDENCE OF CATARACT IN RATS GIVEN GRADED AMOUNTS OF VITAMIN G-CONTAINING FOODS <sup>1</sup>

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ONE FIGURE

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In 1931 a paper from this laboratory (Day, Langston and O'Brien, '31) reported the consistent occurrence of cataract in rats given a diet deficient in vitamin G. More recently (Day and Langston, '34) we reported a larger series of animals, and emphasized the probable significance of cataract as an evidence of G avitaminosis. O'Brien ('32) and Yudkin ('33) likewise found cataract resulting from vitamin G deprivation in a large percentage of animals. Bourne and Pyke ('35), however, obtained only 31% cataract as a maximum in any group of rats. They used a diet essentially the same as ours, but started with much larger animals than we usually employ in vitamin G experiments.

For several years we have been engaged in the assay of various foods for vitamin G. In addition to keeping weekly weight and food consumption records, we have made routine weekly examinations of the eyes of all rats with an ophthalmoscope. The investigator making the eye examinations has always been impressed by a striking relationship between

<sup>1</sup> Research paper no. 502, journal series, University of Arkansas. Aided by grant from the Committee on Live Stock and Meat Board Fellowships of the National Research Council.

failure of growth and appearance of cataract. Negative controls and rats receiving supplements containing very little vitamin fail to grow, and develop an unkempt appearance of the hair. As the experiment progresses such rats exhibit keratitis, and, almost invariably, cataract. Litter mates receiving foods that contain the vitamin grow, and usually fail to develop cataract. It has always been observed that the greater the growth, the less is the probability that cataract will develop. In handling such experimental animals over a period of several years we have become convinced that there must be some fundamental inverse relationship between growth and development of cataract. Furthermore, we have employed a wide variety of natural food substances, and extracts of yeast and liver, and we have never found a substance that would consistently promote growth and fail to prevent cataract, or one that would prevent cataract and fail to promote growth. That is, the growth-promoting and cataract-preventive properties of substances always appear to run parallel, which would lead one to suspect that these properties may reside in the same chemical compound. Such observations are, by their nature, not readily amenable to quantitative expression. We have attempted, however, to tabulate some of our data in such a way as to give a quantitative statement regarding the correlation between growth and incidence of cataract in our experimental animals.

#### EXPERIMENTAL METHOD

The composition of the diet used in this laboratory for the assay of what we shall call vitamin G has been described elsewhere in detail (Day and Langston, '34) and need not be repeated here in its entirety. Young from litters of eight to twelve rats each are placed on experiment when they are 21 days of age, at which time they weigh from 30 to 45 gm. They are given diet 625 in non-scatter food cups, and distilled water *ad libitum*. The diet is made up exactly as described in the previous publication. Since the source and treatment of the casein used may have an effect upon the development

of cataract, a statement should be made regarding the kind we use. We have used 'casein, edible, muriatic,'<sup>2</sup> extracted with 60% alcohol in the laboratory according to the method of Sherman and Spohn ('23). In some experiments we have employed 'vitamin-free' casein.<sup>3</sup> The animals are housed in cages with raised metal floors of  $\frac{3}{8}$  inch mesh, and are maintained at a nearly constant temperature.

After 2 weeks on the basal diet alone, each animal is given a pre-determined amount of a food substance, accurately weighed. This is usually fed either three or six times weekly, depending upon the nature and quantity of the material. One or more animals from each litter are retained as negative controls. The animals receiving addenda are killed at the end of 10 weeks (that is, after they have received the supplements 8 weeks), while the control animals are kept until death intervenes.

Using the foregoing procedure, we have made assays of a number of different substances during the past 2 years. The list of materials fed includes several kinds of cheese, liver extracts, yeast, lamb, pork, pork liver, ham, bacon and other materials.

## RESULTS AND DISCUSSION

If we may assume that the basal diet used furnishes an adequate amount of all necessary growth essentials except one, then the rate of growth of experimental animals receiving such diet should be proportional to the amount of this one substance made available to them. The growth of any animal, then, should be a measure of all the vitamin to which it has access: any reserve bodily store, plus any trace of vitamin in the basal diet, plus any obtained by access to excreta or from other adventitious sources, plus any fed to the animal in a food supplement. In our experiments we have attempted to eliminate, as far as possible, all of the variables except the food supplement. Uniform cages and feeding vessels are

<sup>2</sup> Manufactured by the Casein Company.

<sup>3</sup> Casein Company's Labco brand.

employed, meticulous care is exercised to prevent contamination of the basal diet or cages with vitamin-bearing substances; access to excreta is prevented as far as possible; diet constituents are obtained from constant sources in large quantities, and are given uniform treatment in preparation in this laboratory. All the animals used come from one stock that has received a uniform diet for several generations; only animals from large, healthy, uniform litters are employed in experiments, and they are started on experiment at a definite age when they fall within certain weight limits. With all these precautions, however, there is observed some variation between animals within a single litter, and frequently considerable variation between animals of different litters. Since such differences are frequently manifest in parallel experiments, it is believed that the chief uncontrolled variable in our experiments is the pre-experimental body store of vitamin.

The data from all vitamin G assays over a period of more than a year have been collected and tabulated. All the animals receiving the basal diet only (controls) have been grouped together. The animals receiving vitamin supplements have been grouped according to their growth during the 10-week experiment. Those making net gains in 10 weeks of 20 gm. or less have been placed in group I, those making net gains of 21 to 40 gm. in group II, those making gains of 41 to 60 gm. in group III, and those gaining more than 60 gm. in group IV. It must be admitted that this grouping is quite arbitrary, and that some other division of the animals very probably would have served the purpose just as well.

As an example of the type of supplements fed the animals, one illustration will serve. A series of thirty rats was used in an assay of pork liver. The growth of seven rats receiving 0.05 gm. fresh liver daily placed them in group I. Three rats receiving 0.05 gm. and nine rats receiving 0.1 gm. daily fell in group II. One rat receiving 0.1 gm. daily and three rats receiving 0.2 gm. daily were placed in group III since the total net growth of each in 10 weeks was between 41 and 60 gm. Seven rats receiving 0.2 gm. liver grew more than 60 gm. during the 10 weeks, and so were placed in group IV.

A summary of the data is given in table 1. Of the seventy-eight controls included in this series, only four made net gains of more than 20 gm. during the 10 weeks, and only twenty-three made net gains of more than 10 gm. during the same period. Forty-nine (or 63%) of these control animals exhibited cataractous changes during the 10-week period, and a total of sixty-seven (86%) developed cataract before death. The incidence of cataract in the groups receiving supplements diminished with increasing growth; the four groups showed 39%, 26%, 14%, and 0% cataract, respectively. None of the

TABLE 1

*Table showing incidence of cataract in rats receiving graded amounts of vitamin G-containing foods, grouped according to growth during a 10-week period*

	GROWTH RANGE DURING 10- WEEK PERIOD	MEAN NET GROWTH IN 10 WEEKS	NUMBER OF RATS	CATARACT	
				Number	Incidence
Controls	gm. — 3 to 27	gm. 9	78	49	% 63 <sup>1</sup>
Group I	Up to 20	14	36	14	39
Group II	21 to 40	28	62	16	26
Group III	41 to 60 <sup>2</sup>	50 <sup>2</sup>	28	4	14
Group IV	Above 60	80	28	0	0

<sup>1</sup> Although only 63% of control animals developed cataract in 10 weeks, 86% exhibited cataract before death.

<sup>2</sup> None of the rats growing more than 56 gm. during the 10-week period developed cataract.

animals that made net gains of more than 56 gm. during the period developed cataract, and of forty-seven animals making net gains of more than 30 gm., only seven exhibited cataract. It is thus apparent that cataract was only rarely found in animals receiving enough vitamin to promote growth in excess of an average of 3 gm. weekly.

Figure 1 presents the same data in graphic form, and clearly shows the influence of a very small amount of vitamin on the incidence of cataract. Although the animals in group I made an average gain of only 5 gm. more in 10 weeks than did the control animals, the incidence was much lower—39% as compared with 63% for controls.

The specific type of dermatitis referred to as 'florid' dermatitis by Chick, Copping and Edgar ('35) and photographically shown by Bender et al. ('36) was not observed in any of the animals receiving the diet used in these experiments, although it develops regularly at about 28 days in rats receiving crystalline vitamin B( $B_1$ ) in place of the rice polish extract. This would indicate that our 80% alcohol extract of rice polish contains a considerable amount of the rat dermatitis-preventive factor.

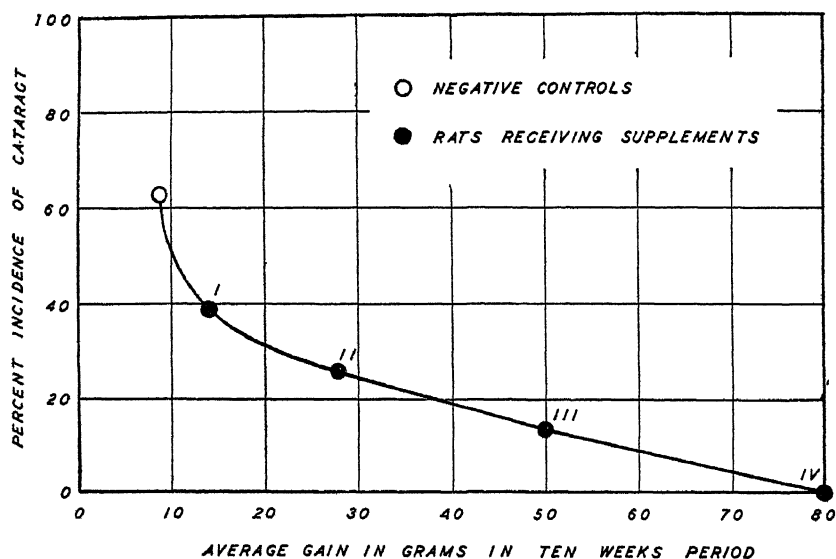


Fig. 1 Chart showing incidence of cataract in groups of rats plotted against average growth. The roman numerals refer to the groups as given in table 1.

Ulceration of the skin was observed occasionally in animals at all levels of growth, and was about as common in animals growing more than 60 gm. (group IV) as in controls or in rats of groups I and II. Roughness and loss of hair was observed in animals of all groups. Definite alopecia was seen in about 35% of the control group and of groups I and II. Loss of hair was less common in rats at the higher levels of growth. Keratitis was observed in practically all animals exhibiting cataract, and in many animals not showing cataract.

Its consistent accompaniment of cataract in these experimental animals leads us to believe that it is certainly a manifestation of the same dietary deficiency that predisposes to cataract.

We are now investigating the possibility of the identity of flavin with the cataract-preventive factor. It appears possible that flavin may have cataract-preventive properties, but we do not yet have conclusive proof that such is the case. However, what is learned in the future regarding the identity or non-identity of flavin and the cataract-preventive substance may slightly alter the interpretation of the results herein reported, but we believe will not detract from their significance.

#### SUMMARY

More than 200 young rats, weighing between 30 and 45 gm., were used in a series of vitamin G assays. For the first 2 weeks they were given a deficient diet only, after which time they were given daily supplements of vitamin-containing foods. Control animals receiving no supplement were kept until death, whereas the animals receiving supplements were killed after 10 weeks.

Eighty-six per cent of seventy-eight controls developed cataract before death, although only 63% showed such eye changes during the 10-week period. The rats receiving vitamin supplement, grouped according to growth during the 10-week period, showed the following incidence of cataract: animals growing 20 gm. or less—39% cataract; animals gaining from 21 to 40 gm.—26% cataract; animals gaining from 41 to 60 gm.—14% cataract; animals gaining more than 60 gm.—0% cataract. It is thus apparent that there was an inverse relationship between growth and appearance of cataract; that is, the greater the growth, the lower the incidence of cataract. This would seem to indicate that, under the conditions of the experiment, growth was a measure of the cataract-preventive property of the supplement. These data also indicate that only a small amount of the vitamin is required to prevent the appearance of cataract.

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# BLOOD SUGAR IN RATS RENDERED CATARACTOUS BY DIETARY PROCEDURES <sup>1</sup>

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ONE FIGURE

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Within the past 5 years it has been shown that cataract may be produced in rats by either of two different nutritional methods. A series of papers from this laboratory (Day, Langston and O'Brien, '31; Langston and Day, '33; Day and Langston, '34) described the consistent occurrence of cataract in albino and Norway rats which had been given a diet deficient in one of the relatively heat-stable factors of the vitamin B complex (vitamin B<sub>2</sub> or G). O'Brien ('32), Yudkin ('33) and Bourne and Pyke ('35) published data corroborating, in part at least, the results obtained in this laboratory.

Mitchell and Dodge ('34, '35) reported the occurrence of cataract in rats given diets containing high percentages of lactose, and later it was shown that galactose produced cataract in a much shorter period (Mitchell, '35; Yudkin and Arnold, '35). It is thus evident that cataract may be produced readily in rats, on the one hand by a deficiency of vitamin G or some closely associated factor, and on the other hand by the presence of large percentages of lactose or galactose in the diet.

It seemed quite possible to the author that the cataract resulting from lactose or galactose feeding might be the manifestation of an abnormal carbohydrate utilization or metabolism. Consequently, the experiments of Mitchell and Dodge

<sup>1</sup> Research paper no. 503, journal series, University of Arkansas.

were repeated, and blood sugar determinations were made upon the animals receiving lactose as well as upon control animals. Preliminary determinations indicated that the lactose rats had elevated blood sugar levels. Experiments were therefore planned to show whether, under carefully controlled conditions, animals receiving high percentages of galactose and lactose would exhibit significant hyperglycemia when compared with animals receiving other common glycogen-forming carbohydrates. Although there was very little reason to believe that an abnormal carbohydrate metabolism would likewise be associated with vitamin G deficiency, it was felt that parallel determinations of blood sugar upon animals developing cataract as a result of vitamin deprivation should be of interest.

#### EXPERIMENTAL METHODS

*Animals receiving normal diets containing various carbohydrates.* Young albino rats from our own stock, weighing 30 to 45 gm., were placed in individual metal cages and fed normal diets containing various utilizable carbohydrates. The composition of the diets was as follows:

	Per cent
Casein, commercial edible	18
Salt mixture, Osborne and Mendel ('19)	4
Cod liver oil <sup>2</sup>	2
Butter fat	6
Yeast, dried <sup>3</sup>	10
Carbohydrate	60

The carbohydrates used were glucose, galactose, sucrose, lactose and starch. The animals in each litter were divided, so far as possible, between the diets containing the different carbohydrates. Weight, food intake, and ophthalmoscopic records were made at weekly intervals.

Blood sugar determinations were made upon blood taken from the tails of the animals at frequent intervals. Sugar was determined by a slight modification of the Folin micro method ('28). Since it was found difficult to obtain even 0.1 cc.

<sup>2</sup> Supplied by the E. L. Patch Company, Boston.

<sup>3</sup> Supplied by the Fleischmann Laboratories, New York.

of blood from the tails of some of the animals, special pipettes calibrated to contain 0.025 cc. were made for the purpose. The blood was collected in these pipettes and diluted with the tungstic acid solution to 2.5 cc. After centrifuging, 2 cc. of the clear filtrate was used for the colorimetric determination, and the final dilution was made in a tube calibrated at 12.5 cc. Only half the usual quantities of reagents were employed; otherwise the technic as described by Folin was followed. Except when diet was withheld for the purpose of making tolerance tests, the rats had access to food at all times. Since young rats eat at frequent intervals, it may be assumed that most of the determinations were made during the absorption of food from the digestive tract.

No attempt was made to determine, qualitatively or quantitatively, the nature of the sugar in the blood. It is assumed, however, that at least a part of the sugar in the blood of rats receiving lactose was galactose, and probably the extremely high values resulting from galactose feeding were due, in part, to the presence of this sugar in the blood. Since galactose is no more strongly reducing toward the reagent used in the Folin micro sugar method than is glucose, the results obtained and here reported should be fairly accurate estimates of the total sugar content of the blood.

On several occasions carbohydrate tolerance tests were run upon groups of the animals, using representative litter mates receiving different carbohydrates. In these tests the diet was removed in the afternoon or evening, and sugar determinations were made early the following morning, after which the rats were given access to a weighed quantity of diet for a period of  $\frac{1}{2}$  hour or 1 hour only. Sugar determinations were then made at hourly intervals during the day. The uneaten residue from each animal's food was weighed to make certain that differences in blood sugar curves were not due to differences in amount of food ingested.

*Vitamin deficient animals.* Young rats were given the vitamin G-deficient diet (no. 625) which has previously been described in detail (Day and Langston, '34). Under the conditions of heredity, age and size of animals, caging, etc., employed

in this laboratory, more than 75% of such animals developed cataract. Litter mates receiving the deficient diet supplemented with dried yeast (as 20% of the diet, replacing corn-starch) were used as normal controls. The experimental and control animals were given access to diet and distilled water at all times, and routine weight, food intake, and ophthalmoscopic records were kept. Blood sugar determinations were made upon deficient and normal animals by tail bleedings, using the Folin method modified as described above. In a few cases 'tolerance' tests were made.

#### RESULTS AND DISCUSSION

Seventeen out of the eighteen rats which were given lactose developed ophthalmoscopic cataract; the average time of appearance was 44 days. In only two cases did the cataracts

TABLE 1

*Incidence of cataract in rats receiving an adequate diet containing various carbohydrates as 60% of the diet*

CARBOHYDRATE FED	NUMBER OF RATS	NUMBER SHOWING CATARACT	TIME OF APPEARANCE OF CATARACT
Glucose	9	0	<i>days</i> ..
Galactose	3	3	11
Sucrose	11	0	..
Lactose	18	17	44
Starch	11	0	..

progress to maturity. The three rats given galactose exhibited ophthalmoscopic cataract on the eleventh day, and mature cataracts on the nineteenth day. This cataract resulting from lactose or galactose feeding was quite different in appearance from the cataract caused by avitaminosis, especially during the early stages of its development. None of the rats that received glucose, sucrose, or starch showed any lenticular changes. The number of animals and incidence of cataract for the various carbohydrates are listed in table 1.

Table 2 gives a summary of data on the blood sugar in rats receiving the various carbohydrates. Since none of the determinations that were made upon rats in the fasting state

have been included in this tabulation, it is believed that the differences were due to the effect of the absorption of food from the intestinal tract. It is evident from a perusal of the table that glucose and starch gave blood sugar levels not significantly different from each other. Rats given sucrose showed slightly elevated blood sugar levels; the average of 133 mg. per 100 cc. blood may be, from a statistical standpoint, significantly greater than the averages for glucose and starch. The blood sugar in rats receiving lactose was extremely variable, values being found all the way from the fasting level up to 234 mg. per 100 cc. The average of eighty-nine determinations was 160 mg. per 100 cc., with a probable error of 2.3 mg.

TABLE 2

*Blood sugar in rats receiving normal diets containing different carbohydrates*

CARBOHYDRATE IN THE DIET	NUMBER OF RATS	NUMBER OF DETERMINATIONS	MEAN BLOOD SUGAR WITH PROBABLE ERROR
			<i>mg. per 100 cc.</i>
Glucose	9	45	121 $\pm$ 1.9
Galactose	3	27	372 $\pm$ 14.0
Sucrose	11	52	133 $\pm$ 2.2
Lactose	18	89	160 $\pm$ 2.3
Starch	11	47	123 $\pm$ 1.8

The probable error of the difference between blood sugar levels of glucose and lactose rats was 3.0 mg., which with a difference of 39 mg., means that the difference is thirteen times the probable error of the difference. If statistical methods may be applied to such observations, the chances are more than 1,000,000,000 to 1 that the difference between mean blood sugar levels in the lactose and in the glucose rats was significant.

Because of the cost of the sugar only three rats were given the galactose-containing diet, but twenty-seven determinations were made upon these three rats. Blood sugars were found to be high and quite variable; one determination showed 556 mg. sugar per 100 cc. of blood. The average of the twenty-seven determinations was 372 mg. per 100 cc., with a probable error of 14 mg. Comparing the average sugar levels in the galactose and glucose animals, and their probable errors, it is

evident that the difference between the two is seventeen times the probable error of the difference. Again, if statistical methods may be applied to such observations, the chances are much more than 1,000,000,000 to 1 that the difference was significant.

A few determinations made upon the experimental animals in the fasting state seemed to show no difference between animals which had been receiving the different carbohydrates. That is, the fasting level of sugar in galactose and lactose rats was not significantly different from corresponding levels of sugar in litter mates receiving other carbohydrates. This might indicate that the lactose and galactose had not caused any permanent derangement in carbohydrate metabolism.

Figure 1 shows graphically the results of a 'tolerance' test, which is typical of those obtained. In this case the diet of all the animals to be used was removed at 4.30 P.M. At 8.30 A.M., after a 16-hour fast, blood was drawn for analysis, and a weighed quantity of diet was given to each rat. After the animals had had access to the diet for 1 hour it was removed, and blood was drawn for analysis at intervals during the day. Within an hour the sugar in the blood of the galactose rat increased from 125 mg. to 532 mg. per 100 cc. It is probable that the peak of the curve was not recorded. At the end of  $2\frac{1}{2}$  hours the blood sugar was falling, and it reached a normal level at the end of  $7\frac{1}{2}$  hours. On the other hand, blood sugar in the animal receiving lactose rose rather slowly, and was still rising at the end of  $7\frac{1}{2}$  hours, at which time it exhibited 200 mg. sugar per 100 cc. blood. The curves of blood sugar for the animals receiving sucrose and glucose were similar to that of the animal receiving starch, and have been omitted from the chart. Speculating on the significance of these curves, it seems apparent that the galactose, requiring no digestion, was absorbed rapidly from the intestinal tract, and yet was only slowly converted into and stored as glycogen. The lactose, on the other hand, required digestion, so that absorption was much slower and the peak of the blood sugar curve was reached much later.

Since the mechanisms operating to cause cataract are very poorly understood, any attempt to explain the production of cataract in these experimental animals in terms of blood sugar level would be largely conjecture. However, there are some comments that may not be altogether inappropriate in discussing the problem. It is interesting that the carbohydrate that

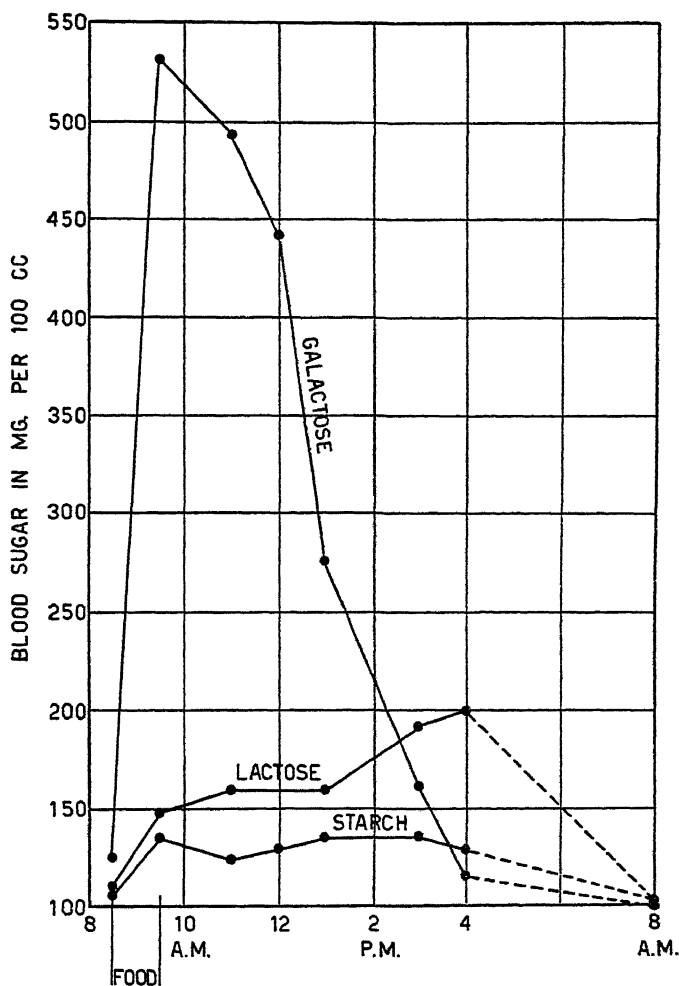


Fig. 1 Blood sugar curves showing tolerance of rats to various carbohydrates. After a preliminary 16-hour fast the animals were given diets containing the carbohydrates indicated on the curves. The animals were given access to a weighed quantity of food for 1 hour, after which it was withdrawn.

caused cataract in the shorter period (galactose) is also the carbohydrate that caused the higher levels of blood sugar. However, even if we were certain that the blood sugar was responsible for the production of cataract, it is of course impossible to state whether it was the level of blood sugar or the specific nature of the sugar that caused the cataract. Kirby, Estey and Wiener ('32) found that galactose was toxic to tissue cultures of lens epithelium at a much lower level than was either glucose or fructose. It may be, therefore, that the cataract was caused by the specific toxic effect of galactose. It is recognized that cataract is a not uncommon complication of diabetes, although it is not known whether it is the hyperglycemia, the lipemia, or some other factor that is responsible

TABLE 3

*Blood sugar in rats receiving a diet deficient in vitamin G and a normal control diet*

	NUMBER OF RATS	NUMBER OF DETERMINATIONS	MEAN BLOOD SUGAR WITH PROBABLE ERROR
Deficient diet	21	40	<i>mg. per 100 cc.</i> 107 $\pm$ 1.2
Normal diet	9	38	118 $\pm$ 1.4

for the development of diabetic cataract. It is possible that diabetic cataract, and experimental cataract resulting from galactose feeding, have a common mechanism of formation.

Table 3 summarizes the data upon blood sugar in vitamin G deficient animals and normal controls. There was no increase in blood sugar as a result of the avitaminosis. There was, in fact, a slightly lower sugar level in the blood of rats on the deficient diet, which may be statistically significant. It probably was due to partial inanition, and it is difficult to see how it could have been related to the formation of cataract.

Since cataract following lactose or galactose feeding differs in ophthalmoscopic appearance from cataract following vitamin withdrawal, it seems likely that the two types result from different metabolic disturbances.



## SUMMARY

Young growing rats were given adequate diets containing various carbohydrates as 60% of the diet. Blood sugar was determined by the Folin micro method. Seventeen out of eighteen rats receiving lactose developed cataract at an average time of 44 days. The three rats receiving galactose exhibited cataract on the eleventh day. Glucose, sucrose and starch did not cause cataract.

The mean blood sugar level in rats receiving glucose ad libitum was 121 mg. per 100 cc. The level in rats receiving starch was essentially the same, but was slightly higher in rats receiving sucrose. Lactose and galactose caused high and extremely variable blood sugar levels. The mean of eighty-nine determinations upon lactose rats was 160 mg.; the highest value found was 234 mg. The mean of twenty-seven determinations upon galactose rats was 372 mg.; the highest level recorded was 556 mg. Considered statistically, the chances are more than 1,000,000,000 to 1 that these mean values are significantly greater than the corresponding mean value for rats receiving glucose. It may be significant that the sugar that caused cataract in the shorter period (galactose) also produced the highest blood sugar levels.

Cataract was produced in young growing rats by a diet deficient in vitamin G. The mean of blood sugar determinations upon such rats was somewhat lower than the mean blood sugar of normal controls.

The cataract produced by lactose or galactose feeding was easily distinguished with the ophthalmoscope from cataract resulting from vitamin deficiency.

The author is indebted to Mr. William J. Darby for assistance in care of the animals and to Dr. K. W. Cosgrove for making slit-lamp examinations of eyes of some of the animals.

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# COMPARISON OF THE BIOLOGICAL AND CHEMICAL METHODS FOR THE DETERMINATION OF VITAMIN C IN CANNED STRAINED VEGETABLES AND A STUDY OF ITS VARIATION FROM YEAR TO YEAR<sup>1</sup>

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For 5 years, we have studied the vitamin C content of canned strained vegetables of one brand: tomatoes, spinach, peas and green beans. For the first 2 years, the biological test on guinea pigs was used; then, for 2 years, the biological test was compared on the same foods with the chemical method of titrating with sodium 2:6 dichlorobenzene indophenol; and, in 1935, the chemical method alone was used. This series of tests then gives data upon which to compare the two methods of determining vitamin C in these products and to note the variation in vitamin C potency from year to year.

## I. COMPARISON OF METHODS

A chemical method, such as titrating the reducing value of an extract of the vegetable with sodium 2:6 dichlorobenzene indophenol, is more sensitive and much less expensive and time consuming than the biological test. However, the titration method is not specific for vitamin C and hence must be carefully controlled and checked for its reliability. We used this method, at first, as a guide in choosing the quantities to be fed the guinea pigs, since the biological tests on such products in 1930 and 1932 showed great variation.

<sup>1</sup> Financed by a grant from the Gerber Products Co.

The volumetric method of Bessey and King ('33) was used with two minor changes. Instead of three grindings of the food with sand and 10% acetic acid, only one grinding was necessary for the strained foods, but four additional washes were made. Since many of the extracts were colored and the red color of the dye difficult to see, a distinct end point was made possible by choosing a pink color stable for 10 seconds as the end point and by comparing it with two blanks: one of the extract as titrated, and another to which 1 or 2 drops of methyl red had been added to give a stable color similar to the end point.

To determine whether this method was a reliable one for canned foods, we studied its accuracy and possible errors and compared the chemical and biological methods on eleven samples of canned strained vegetables. Each sample was analyzed in triplicate or quadruplicate. In a series of samples, a solution of pure vitamin C was added at the beginning of the analysis to one of the aliquots. From the titration values of the aliquots and of the solution of vitamin C, the per cent of recovery was calculated. The results averaged 95.75%, ranging from 91.2 to 97.2%. It was thought possible that iron and tin salts dissolved from the can would interfere with a true test. However, the addition of the maximal amounts of tin and iron salts found in canned foods to some of the aliquots being titrated did not change the values. Concentrations of oxalic acid as found in spinach, likewise, did not affect the results. Aliquots from the same sample ordinarily checked within 3 to 5% error, even when small amounts of vitamin C were present. The method, thus, could be considered of greater sensitivity and accuracy than a method based on an animal response, but whether vitamin C was truly measured needed to be checked by comparison with the biological method.

The biological method of Sherman, LaMer and Campbell ('22) was used with an addition of 2% dried baker's yeast and 2% cod liver oil to the basal diet. The data of all animals which consumed the supplements are included in the averages. Some of the tests were exploratory in nature and only a few

animals used. The control animals, those fed cabbage or lemon juice or the basal ration only, were quite uniform from year to year, as shown in table 1. As the International Standard, 1 cc. lemon juice contains 10 units and gives an average scurvy score of 1 to 2. The vegetable supplement giving scurvy scores approximately the same would contain 10 units, or a score of 0, as from 1.5 cc. of lemon juice, indicates 15 International units and may be used for comparisons also.

To compare the values obtained by the chemical and biological methods, the analytic data from the titration values

TABLE 1  
*Record of control animals in the biological vitamin C determinations*

SUPPLEMENT	YEAR	AMOUNT FED	NUMBER OF ANIMALS	AVERAGE INITIAL WEIGHT	AVERAGE GAIN IN WEIGHT	AVERAGE SURVIVAL TIME <sup>1</sup>	AVERAGE SCURVY SCORE	RANGE OF SCORE
		cc.		gm.	gm.	days		
None	1930		6	316.2	— 99.0	30	15.0	9-17
None	1932		5	299.8	— 97.6	29	13.4	7-21
None	1933		5	329.0	— 86.0	27	13.6	10-17
None	1934		6	321.0	— 109.8	25	15.8	10-21
Raw cabbage	1930	Ad lib.	4	301.5	+ 312.0	90	0	0
Lemon juice	1932	1.5	3	282.3	+ 255.0	90	0	0
Lemon juice	1932	1.0	7	329.0	+ 172.6	90	1.2	0-4
Lemon juice	1933	1.0	9	310.3	+ 153.8	56	1.8	0-3
Lemon juice	1934	1.0	9	289.0	+ 154.8	56	1.4	0-4

<sup>1</sup> The experimental test period of 1933 and 1934 was limited to 56 days, but for 1930 and 1932 was 90 days.

of the vegetables of the years 1933 and 1934 were used in calculating the amounts to be fed the guinea pigs. A series of lemons were tested and found to contain on the average 0.565 mg. vitamin C per cubic centimeter, equivalent to 10 International units. The guinea pigs were fed sufficient of each vegetable to give 0.565 mg. vitamin C, based on its titration value. The data are presented in table 2. It will be noted that of the eleven samples from the 2 years the average scurvy scores for the vegetable supplements ranged from 0.7 to 2.4 while those of 1 cc. lemon juice in table 1 were 1.2 to 1.8.

Considering deviations found in the chemical method, the usual biological variations of the animals and the more or less qualitative scoring of the degree of scurvy, the data indicate that the two methods give reasonably concordant results. Since the chemical method gave the same result as the biological, the vegetables canned in 1935 were tested chemically only.

TABLE 2

*Record of animals fed vegetable supplements containing equal quantities of vitamin C<sup>1</sup>*

SUPPLEMENT	YEAR	AMOUNT FED	NUMBER OF ANIMALS	AVERAGE INITIAL WEIGHT	AVERAGE GAIN IN WEIGHT	AVERAGE SCURVY SCORE	RANGE OF SCORE
		<i>gm.</i>		<i>gm.</i>	<i>gm.</i>		
Tomatoes	1933	4.1	7	340.4	+ 96.6	0.7	0-2
Tomatoes A		4.1	7	336.7	+ 123.7	1.0	0-4
Tomatoes B		4.3	8	290.9	+ 180.9	1.1	0-3
Tomatoes C		9.0	8	294.3	+ 137.0	2.3	0-6
Spring spinach <sup>2</sup>	1933	4.3	7	301.7	+ 131.6	1.6	1-2
Spring spinach	1934	5.7	7	308.4	+ 112.7	2.4	1-4
Fall spinach	1934	3.4	9	301.0	+ 154.9	2.3	1-5
Peas	1933	6.3	8	317.3	+ 239.8	1.4	0-3
Peas	1934	8.5	8	308.4	+ 181.0	1.9	0-4
Green beans <sup>2</sup>	1933	12.5	5	300.2	+ 147.9	2.2	2-3
Green beans	1934	17.8	8	317.8	+ 195.4	2.0	1-3

<sup>1</sup> These vegetables were first tested chemically and the quantities fed calculated from that data to contain 0.565 mg. vitamin C.

<sup>2</sup> The animals receiving spinach and green beans had to be coaxed and sometimes fed by hand but the supplements were completely consumed.

## II. VARIATION IN VITAMIN C CONTENT FROM YEAR TO YEAR

The samples used for all these tests were representative of the entire season's pack except for tomatoes A, B and C which were special samples and for the 1930 vegetables which were chosen from the warehouse. These foods were canned by the Gerber Products Co. who dated and set aside cans from each day's pack. An equal number of cans of each date were taken to form the twenty-four to sixty cans required for a representative sample. The error in sampling was, thus, practically eliminated.

The results of determinations, both chemical and biological, are presented in table 3 and calculated in terms of International units per ounce. The unit value is determined in the

TABLE 3

*The variation in vitamin C content of canned strained vegetables from year to year*

SUPPLEMENT	YEAR	TOTAL SOLIDS <sup>1</sup>	NUMBER OF ANIMALS	AMOUNT FED	AVERAGE SCURVY SCORE	NUMBER OF SAMPLES FOR CHEMICAL DETERMINATION	TITRATION VALUE	VITAMIN C
		%		gm.			mg. vitamin C. per gram	International units per ounce <sup>2</sup>
Tomatoes	1930	12.30	5	3.0	0.0	0		142
Tomatoes	1932	14.80	4	2.0	13.8	0		†*
Tomatoes	1933	13.96	7	4.1	0.7	4	0.137	70
Tomatoes	1935	12.27	0			4	0.129	64
Spinach	1930	5.60	2	12.0	1.5	0		24
Fall spinach	1931	6.93	6	16.0	0.0	0		27
Spring spinach	1932	6.59	6	16.0	0.8	0		18
Spring spinach	1933	7.89	7	4.3	1.6	5	0.132	66
Spring spinach	1934	7.27	7	5.7	2.4	4	0.099	50
Fall spinach	1934	7.47	9	3.4	2.3	4	0.167	85
Spring spinach	1935	6.72	0			2	0.104	53
Peas	1930	12.10	4	8.0	2.4	0		35
Peas	1932	12.71	4	8.0	0.0	0		53
Peas	1933	14.47	8	6.3	1.4	4	0.090	45
Peas	1934	13.44	8	8.5	1.9	4	0.066	33
Peas	1935	13.38	0			4	0.063	32
Green beans	1930	6.08	2	16.0	4.0	0		< 17
Green beans	1932	6.81	6	16.0	0.7	0		17
Green beans	1933	7.45	5	12.5	2.2	3	0.045	23
Green beans	1934	6.56	8	17.8	2.0	4	0.032	15
Green beans	1935	6.33	0			4	0.018	9

<sup>1</sup> Determined in a vacuum oven at 70°C. except for fall spinach of 1931 and 1934 which were air dried in an electric oven at 100°C. to constant weight. Attention is called to the fact that while the total solids content of the canned strained spinach was approximately the same as for raw spinach, the tomatoes contained twice and both peas and green beans about one-half the average total solids content of raw vegetables.

<sup>2</sup> These values are based on either biological test when no titration value was determined or upon the chemical method when the titration value is given.

<sup>3</sup> This value was too low to be calculated since none of the control animals receiving lemon juice had scurvy scores approximating this. The animals did live through the experimental period while the negative controls with similar scores did not.

biological test by comparison with the scurvy scores of guinea pigs fed lemon juice or, in the chemical method, by the amount which from the titration data contains 0.0565 mg. of vitamin C.

The potency of these products varies markedly from year to year as noted in the last column of table 3. Tomatoes were high in vitamin C in 1930 but very low in 1932, for which we have no explanation except that of seasonal variation in the raw product. None were canned in 1934 since the raw product was inferior. The canning method was greatly changed in 1933 by using a new type of cooker for evaporation, and in 1935 a marked reduction in the time of heating. The tomatoes contained 70 International units vitamin C per ounce in 1933 and 64 in 1935. Thus, there was no increase in vitamin C content due to the change in method which the seasonal variation in raw tomatoes did not mask.

Spinach has been especially rich in vitamin C for the last 3 years, particularly in the fall of 1934, and shows a marked improvement over that of the 3 earlier years. A steam blanch has been used since 1933 and the evaporation time reduced. The 'shaker-cooker' process was introduced in 1934. Spinach was found to vary from 18 to 27 International units vitamin C per ounce from 1930 to 1932 but 50 to 85 units from 1933 to 1935.

Peas showed a variation in vitamin C content from 32 to 53 International units per ounce. The total solids content has been increased since 1933 by greater evaporation and the 'shaker-cooker' used for the final heating since 1934. It is interesting to note that peas are more uniform in vitamin C from year to year than the other vegetables.

Green beans are a fair source of vitamin C, varying from 9 to 23 International units per ounce. The 'shaker-cooker' process was introduced in 1934. The method was not changed from 1934 to 1935 yet the last year's pack was low in vitamin C.

A study of the variation in vitamin C content in these four vegetables shows that it is difficult to indicate which factors had the greatest effect on their potencies. Since all of these vegetables during the last 4 years were carefully chosen as



representative samples, the error of sampling is practically nil. There were some differences in total solids content from year to year but they are not significant in explaining the vitamin variation. The soil conditions were fairly uniform and there were very few changes in varieties of plants. Although the raw foods were carefully graded as to appearance and degree of maturity, there seems to be an important seasonal variation.

Another factor which may influence the final antiscorbutic potency is that of factory procedure. The method used depends on exclusion of air by a blanket of steam or closed container and has been modified, as previously indicated, in several ways to reduce heating time. The factory method has been gradually improved from year to year so that we should expect any losses of vitamin C to be steadily reduced. Although the data do not clearly distinguish the effect of the two factors, the original variation in raw foods due to weather conditions and the losses in the factory process, yet it seems to us that, in this study, the seasonal variation is the more important.

It is evident that predictions of potency from 1 year's test may be far different from that actually found in another year, and that cans chosen from the grocer's shelf may vary widely in vitamin C potency. The producer of canned strained vegetables must be diligent in considering all the factors affecting their vitamin C potency and carefully controlling those within his power in order that a product as rich as possible in vitamin C be placed upon the market. The standardization of such potency, however, from the standpoint of the manufacturer, presents a difficult problem.

#### SUMMARY

The determination of vitamin C in canned strained vegetables by titration with sodium 2:6 dichlorobenzenone indophenol has been found accurate and reasonably concordant with biological tests on the same samples.

Marked variations in vitamin C content have been found in tomatoes, spinach, peas and green beans from year to year.

A discussion of the factors affecting the quality of the vegetables leads to the conclusion that the standardization of the vitamin C potency even under carefully controlled conditions is a difficult problem.

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# STORAGE OF VITAMIN C BY NORMAL ADULTS FOLLOWING A PERIOD OF LOW INTAKE <sup>1</sup>

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## ONE FIGURE

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It is recognized that the human body has some ability to store vitamin C (Yavorsky, Almaden and King, '34; Johnson and Zilva, '34), therefore the apparent requirement at any given time will depend in part on the store of vitamin C in the tissues. It is also recognized that the urinary excretion of ascorbic acid is dependent upon at least two factors: 1) the dietary intake and 2) the reserves of vitamin C in the tissues (Johnson and Zilva, '34; Harris and Ray, '35). With this in mind it seemed desirable to investigate the urinary output of persons in a known state of saturation with respect to vitamin C.

Since it has been reported (Göthlin, '31; Dalldorf and Russell, '35) that capillary resistance varies with the vitamin C intake, it was hoped that change in capillary resistance would serve as an indication of vitamin C subnutrition in this study.

## EXPERIMENTAL

*Urinary excretion.* This experiment was planned to determine the vitamin C intake necessary to reestablish saturation after prolonged administration of a low C diet.

<sup>1</sup> The manuscript from which these data were taken was submitted by Patricia H. O'Hara in partial fulfillment of the requirements for the degree of master of science, 1936. The authors wish to express their appreciation to Dr. Helen Bull, who made the medical examinations, and to the subjects without whose cooperation the study would have been impossible.

The vitamin C content of foods and urine specimens was determined by indophenol titration essentially according to the method of Bessey and King ('33). Ten cubic centimeters of urine were diluted to 50 cc. with 8% acetic acid, in a volumetric flask, and 10 cc. of this solution were titrated rapidly against a standard solution of 2-6 dichlorophenolindophenol from a microburette. Preliminary trials indicated that a more satisfactory end point was obtained, especially in dilute solution, by this method than when the reverse titration was employed as suggested by Birch, Harris and Ray ('33).

During the day time urine specimens were titrated immediately after voiding. The evening and first morning specimens were preserved by acidifying with 10% glacial acetic acid as suggested by Harris and Ray ('35) and titrated in the morning. Titrations made on the same specimen when freshly voided and after preserving in this way from 2 to 12 hours, confirm the observation of Harris and Ray that little loss occurs. In addition, since most of the vitamin C ingested in this experiment was ingested with the morning meal, and since the peak of excretion was reached in 4 to 6 hours after ingestion of a large dose of vitamin C, the error introduced by preserving the evening and first morning specimens was certainly small and does not invalidate the results of the study.

In a preliminary experiment the two subjects were saturated as a result of the inclusion of liberal amounts of orange juice in the diet. Weekly determinations on their usual diets indicated an average excretion of about 70 and 77 mg. of ascorbic acid, respectively.

On the basal diet which provided approximately 11 mg. of ascorbic acid per day, the urinary excretion fell to an average of 11 and 17 mg. during the second week. When orange juice was again included in the dietary approximately 700 and 900 mg. of ascorbic acid were ingested, over a period of 8 to 9 days, before any rise in urinary excretion occurred. This experiment was discontinued before the subjects reached saturation.

In the present experiment, four normal adult women were used as subjects. Titrations made previous to the experiment while the subjects were on their usual diets, indicated that their tissue reserves of vitamin C were satisfactory, since the average urinary excretion of ascorbic acid ranged from 65 to 77 mg. per day.

The basal diet was planned to be adequate with respect to all other nutritive factors, but contained only 5 mg. of vitamin C. The following foods were used ad libitum: a whole wheat cereal with added wheat germ, butter, brown sugar, Ry Krisp, whole wheat and white flour, nuts, cooking fat, eggs, rice, coffee and postum. The following were included in definite amounts each day, either because they contained traces of vitamin C, or because a certain intake was considered advisable to insure the adequacy of the diet:  $\frac{1}{4}$  cup evaporated milk, 2 ounces American cheese, 100 gm. canned carrots, 50 gm. cooked dried prunes, 100 gm. cooked frosted squash, 100 gm. canned pears, 100 gm. hamburger steak. The approximate average composition of the basal diet, as estimated from tables of food composition was as follows: protein, 83 gm.; calcium, 1.04 gm.; phosphorus, 1.50 gm.; iron, 12 mg.; calories 2240. The orange juice additions were titrated daily, immediately before the dose was given.

The experiment was divided into three periods as follows:

I. A preliminary period (4 to 5 days) to be sure that all subjects were saturated. In addition to the basal diet orange juice of known potency was ingested as indicated in figure 1.

II. Basal diet only (29 to 30 days).

III. Basal diet plus orange juice with an ascorbic acid content of 200 mg., taken at breakfast. This period was continued until the subjects were saturated, as evidenced by failure to show further increases in urinary output of vitamin C on a constant high intake (15 to 17 days).

*Capillary resistance studies.* Capillary resistance was determined weekly, alternately on the right and left arms, by Göthlin's method ('31) and daily by means of the resistometer devised by Dalldorf (Dalldorf and Russell, '35).

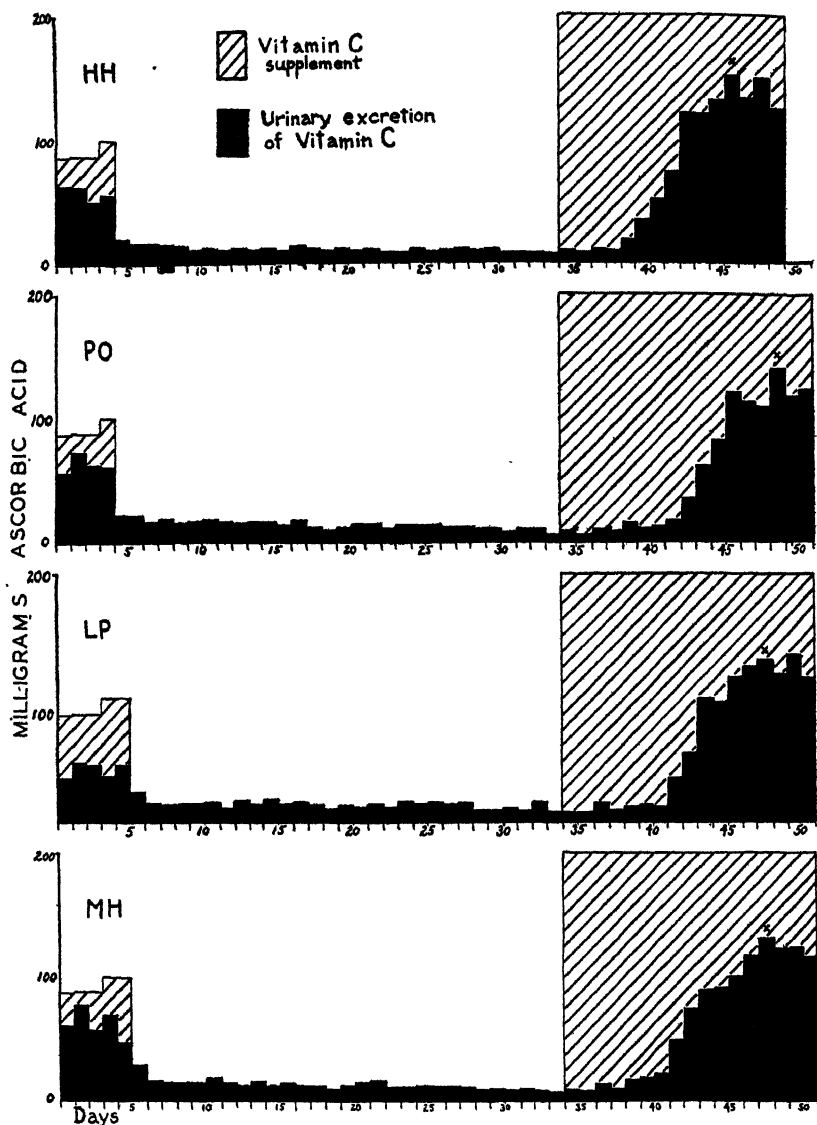


Fig.1 This figure shows the relation of daily intake of vitamin C to the 24-hour excretion of ascorbic acid. A basal diet providing 5 mg. of vitamin C was used throughout. X indicates the day on which the subject was considered to be saturated when a vitamin C supplement was resumed after a period of deprivation.

In consideration of the factors which Göthlin ('33) has found to influence capillary resistance, all tests were carried out at comfortable room temperature, after the subjects had been indoors for an hour or more. Subjects H.H. and P.O. took a warm shower each morning, while the other subjects took tub baths in the evening. All tests were carried out between 9 A.M. and 2 P.M.

Göthlin's standard ('31) for judging the response was used; the resistance was considered normal when less than five petechiae occurred at a pressure of 50 mm. mercury, and sub-normal when more than eight occurred at this pressure, or when two or more petechiae appeared at 35 mm. pressure.

In determining capillary resistance with the Dalldorf resistometer, tests were made in an area  $3\frac{1}{2}$  inches square, on the right outer arm, midway between shoulder and elbow. Twenty-five tests could be made in this area, so that a given space would not be retested for 5 or 6 days.

In reading the test, the surrounding tissue was blanched by pressing with a plane glass, thus bringing petechiae into prominence. All readings were made by a daylight lamp.

On days when the Göthlin test was made on the right arm, the determination was made first by the method of negative pressure to avoid possible influence of the venous stasis produced during the Göthlin test.

In the preliminary experiment, readings made with Dalldorf's resistometer were considered positive if a single hemorrhage was observed. The readings so obtained were consistently lower than those reported by Dalldorf and Russell ('35) for well-nourished persons. Later,<sup>2</sup> it was decided to adopt as the threshold reading the lowest negative pressure which, on casual inspection, gave an immediate impression that bleeding had occurred.

In addition to the impression as to whether a reading was 'positive' or 'negative,' the space tested, the number and size of petechiae, and the appearance of the area on the following day were recorded. The readings which were considered positive were characterized by the appearance of several petechiae

<sup>2</sup> On the basis of a conference with Dr. Gilbert Dalldorf.

(about 80% included seven to twenty-five petechiae) distributed through the test space (not confined to the border), producing a discolored area which was apparent the following day. As a rule, in readings which were considered negative, there were from none to ten petechiae.

#### RESULTS AND DISCUSSION

*Urinary excretion studies.* Figure 1 shows the dietary intake and urinary excretion of ascorbic acid by the four subjects.

It has been shown (Abassy, Harris, Ray and Marrack, '35) that the non-specific reduction titre, due to substances other than vitamin C, does not normally rise above an equivalent of 3 to 6 mg. per day. In the accompanying tables, the values are uncorrected.

It will be noted that the subjects were 'saturated' at the beginning of the experiment, i.e., on an intake considerably higher than the estimated requirement, the subjects maintained a relatively constant output. If unsaturated, the subject requires several days to reach a relatively constant output (Johnson and Zilva, '34). During the period on the basal diet only, the urinary output of ascorbic acid fell sharply, reaching a low level of 15 mg. in 1 to 2 days.

It is of interest that this fall in urinary excretion of vitamin C occurred less rapidly and was less marked in the preliminary experiment, in which the basal diet provided approximately 11 mg. of ascorbic acid per day, than in this experiment when the basal diet contained approximately 5 mg. of ascorbic acid. It is possible that the continued excretion of the usual amounts of ascorbic acid which has been observed by some workers (Harris, Ray and Ward, '33; Hawley, Stephens and Anderson, '36) may be due to the use of a basal diet more liberal in vitamin C. Hawley, Stephens and Anderson estimated the ascorbic acid content of their low vitamin C diet as between 10 and 20 mg. per day.

Table 1 summarizes the ascorbic acid excretion during the preliminary saturation period and the period of vitamin C



deprivation. All subjects showed a general tendency to decreasing excretion of ascorbic acid as the period on the basal diet only progressed. Indeed this tendency continued for a few days after the orange juice supplement was resumed. At the end of a month on the basal diet all subjects continued to excrete indophenol reducing substance over and above the 3 to 6 mg. estimated by Abassy, Harris, Ray and Marrack ('35) as representing the non-specific titration. It was not, of course,

TABLE 1

*Summary of ascorbic acid excretion on a diet low in vitamin C, following saturation*

PERIOD	DAYS OF PERIOD	ASCORBIC ACID IN URINE							
		H.H.		P.O.		L.P.		M.H.	
		Range	Average	Range	Average	Range	Average	Range	Average
I Ascorbic acid supplement 88-100 mg.	1-4 or 5	mg. 50-63	mg. 56	mg. 56-72	mg. 62	mg. 34-48 <sup>1</sup>	mg. 42	mg. 46-77 <sup>1</sup>	mg. 61
II Basal diet	1		19		21		24		28
	2- 8	11-15	13	14-21	16	11-17	15	12-16	14
	9-15	9-13	11	10-16	14	11-18	14	10-14	12
	16-22	8-11	9	11-14	13	12-15	13	11-15	12
	23-29	7-11	9 <sup>2</sup>	6-12	10 <sup>2</sup>	9-14	11	7-11	9
	Or 30								

<sup>1</sup> Five-day period.

<sup>2</sup> Thirty-day period.

intended that the experiment should continue until clinical signs of scurvy should appear.

That the bodily stores of vitamin C were nearing depletion is suggested by the fact that in one subject (H.H.) the gums on the left side of the mouth, on both upper and lower jaws from the first premolars through the second molars were swollen, edematous and purple. Since this occurred during the fourth week on the low C diet, and disappeared quite promptly after orange juice was administered, it may probably be interpreted as a symptom of vitamin C subnutrition.

All subjects were examined by a physician at the end of the second period. Particular attention was paid to examination for symptoms of latent scurvy: tenderness over the long bones, edema and hemorrhage of the gums and roof of mouth, abnormal heart sounds, hemorrhages in skin and conjunctiva, and red blood cells in the urine. With the exception of the gingival bleeding observed in the subject H.H., no clinical symptoms suggestive of vitamin C subnutrition were found. It is of interest that in this subject, the urinary excretion of vitamin C fell more rapidly on the basal diet than in the other subjects (fig. 1) reaching a relatively constant low level of excretion in the third week. Furthermore this subject showed a more rapid rise in excretion when vitamin C was again included in the diet, and reached saturation earlier than the other subjects. Whether this difference was due to the smaller body size of the subject H.H. or to an individual difference in capacity to store the vitamin, or to some other factor, it is impossible to conclude from these experiments.

During saturation on the 200 mg. supplement, the average variation in urinary excretion from that which occurred on the day saturation was reached was only 11 mg., or less than 6%. The maximum variation during saturation ranged from 7.5 to 13% for the four subjects.

There was a general tendency for the difference between intake and urinary excretion to be greater when equilibrium was established on an intake of 200 mg. than on the lower intake of the first period. At present all of the factors which cause this difference between intake and urinary excretion are not known. In addition to storage, it may represent failure of absorption, excretion by paths other than the kidney, destruction in the bladder, chemical changes during metabolism and other factors.

However, unless some ability to synthesize vitamin C is assumed, one may say that the amount of vitamin C stored on a given day cannot be more than the difference between intake and urinary output. Therefore a study of these differences, following vitamin C deprivation, up to the time saturation is reached, should give some indication of possible storage.

Table 2 summarizes the ascorbic acid excretion when orange juice was given, following a period of vitamin C deprivation. From 1000 to 1600 mg. were ingested before a noticeable increase in excretion occurred. From 2200 to 2800 mg. had been taken before saturation was reached. Of this, from 1710 to 2181 mg. was not accounted for in the urine. It seems probable that a large part of this difference was stored. It has been shown that the store of vitamin C in the guinea pig liver is reduced to about one-third when the guinea pig begins to lose weight on a scorbutic diet, before manifest symptoms of scurvy

TABLE 2

*Summary of urinary excretion of ascorbic acid, in relation to intake, following Vitamin C deprivation*

SUBJECT	H.H.	P.O.	L.P.	M.H.
From beginning of period to first distinct increase in ascorbic acid excretion				
Number of days	5	8	8	6
Total amount supplement, mg.	1000	1600	1600	1200
Total ascorbic acid output, mg.	57	95	120	75
Difference between intake and output, mg.	943	1505	1480	1125
From beginning of period to saturation				
Number of days	11	14	13	13
Total amount supplement, mg.	2200	2800	2600	2600
Total ascorbic acid output, mg.	490	619	617	620
Difference between intake and output, mg.	1710	2181	1983	1980

appear (Yavorsky, Almaden and King, '34). If we assume a similar store remaining in the tissues of these subjects after a month on a low C diet, the resulting estimation of the storage of these subjects at saturation would be in the neighborhood of 2500 to 3000 mg.

It is unfortunate that of the considerable number of studies on urinary excretion of ascorbic acid in the literature most yield no information on the amount of ascorbic acid which might represent storage at saturation, either because nothing was known of the state of vitamin C reserves at the outset, or the experiments were not carried on to saturation, or the doses were varied so that the probable date of saturation could not

be estimated, or because the data on urinary excretion were incomplete.

Johnson and Zilva ('34) found that a man who had been on a winter diet (England) and whose excretion of ascorbic acid was so low as to be almost negligible, reached saturation when 1485 mg. of ascorbic acid had been ingested. Of this amount 1141 mg. did not appear in the urine. Although the variation in daily dose makes it difficult to know just when their subject A.E.K. became saturated, these authors found that saturation occurred more quickly on a high intake—apparently after 3649 mg. had been ingested during a 4-day period. Of this 2363 mg. did not appear in the urine. Harris and Ray ('35) had one subject with a daily excretion of about 20 mg. of vitamin C on his usual diet, whose ascorbic acid excretion was followed after 8 days on a vitamin C free diet until he became saturated. In the course of a 25-day period (including 7 days on a low C diet) this subject ingested 3500 mg. of ascorbic acid, of which 1060 appeared in the urine, leaving a balance of 2440 mg. unaccounted for.

The excretion during a period on a diet low in vitamin C is not an indication of the extent to which body stores are being depleted, since in the experiments here reported, the difference between vitamin C intake and excretion up to the time saturation was reached, was more than five times the ascorbic acid excretion during the low C period which preceded.

In addition to the studies on living subjects, the study of Yavorsky, Almaden and King ('34) on tissues obtained at autopsy give a clue to the storage of vitamin C in the body. On the basis of their figures for the average vitamin C content of human tissues (adrenal, brain, heart, liver, pancreas, kidney, lungs, spleen), the average store of vitamin C in the adult would be in the neighborhood of 850 to 950 mg. However, these authors state that the maximum concentration found was approximately three times the average, which would lead one to expect a maximum storage of approximately 2500 to 2850 mg. These figures agree closely with the estimated storage on the basis of the present study.

It is of interest to see how the data obtained in this experiment fit the several methods which have been suggested for estimating the state of the vitamin C reserves in the human body. Archer and Graham ('36) believe that special significance attaches to the amount of ascorbic acid which must be taken before the output rises above 75%. In the case of the four subjects here reported, the average percentage output ranged between 53 and 72 during saturation at different levels of intake. On only 1 day, did the excretion for any subject rise to 75% during saturation on the 200 mg. level; the average excretion for the four subjects on this level varied from 62 to 70% of the intake. During the preliminary saturation period two other subjects reached an excretion of over 75% on 1 day each.

Harris and Ray ('35) state that with the usual volume of urine, an adult excreting 0.02 to 0.03 mg. per cubic centimeter per day may be safely assumed to have adequate vitamin C reserves, although these authors consider the response to a large test dose to be a better criterion.

Ippen ('35) suggests that a rise in urinary excretion to more than 0.04 mg. per cubic centimeter after a large test dose may be used as an indication of satisfactory reserves of vitamin C.

In the present studies the ascorbic acid excretion fell below 0.02 mg. per cubic centimeter per 24 hours within a day or 2 after the low vitamin C period was begun and did not return to this level until 5 to 9 days after the 200 mg. supplement had been added. At this time the total daily urinary excretion of vitamin C ranged from 18 to 36 mg. for the four subjects. The concentration of vitamin C in the urine following the ingestion of a 200 mg. dose rose to 0.04 mg. per cubic centimeter at the height of the excretion either coincidentally with or the day following that on which the total daily excretion reached 0.02 mg. per cubic centimeter. Thus it appears that these two criteria measure a similar state of vitamin C reserve, and that they may be met when the body falls considerably short of saturation.

*Capillary resistance studies.* The results of the capillary studies for a consecutive period of 14 to 17 weeks are summarized in table 3. It is apparent that there were marked variations in response to the capillary resistance tests, not only in different individuals, but in the same subjects from time to time. There was more variation between maximum and minimum readings obtained by the Dalldorf method during a given period, than between the average readings for the several periods.

Frequently both negative and positive readings were obtained at the same pressure in successive tests on the same day. Of ninety-four such tests on the four subjects, seventy-seven or 82% of the negative readings occurred in the two-fifths of the area nearest the elbow. In general, it appeared that higher pressures were required to produce hemorrhage in the spaces toward the elbow, than those toward the shoulder.

With Göthlin's test, some borderline and subnormal readings were obtained in all subjects on their usual diets. These were particularly frequent in subject H.H., who, judging from the urinary excretion of ascorbic acid on her usual diet, must have had satisfactory tissue reserves. Of eighteen subnormal tests obtained in fifty-eight determinations by Göthlin's method on the four subjects, eight occurred during the period of low vitamin C intake, five during the recovery period (three when the subjects were saturated or nearly so) and five on the subject's usual diets. Of a total of sixteen tests made during the period on the basal diet, nine were normal, eight subnormal and three borderline according to Göthlin's standard.

Of twelve tests made during menstrual periods, six gave subnormal values. One of these occurred during the period of vitamin C deprivation.

No correlation was found between the results obtained by the two methods. Subjects L.P. and M.H., whose capillary resistance as judged by the Dalldorf test tended to be low, had fewer subnormal or borderline tests as judged by Göthlin's technic than the other subjects. Many examples of lack of correspondence between the results of the two tests were

**TABLE 3**  
*Relation of capillary resistance to vitamin C intake and excretion*

DIET AND PERIOD	H.H.				P.O.				L.P.				M.H.			
	Capillary resistance				Capillary resistance				Capillary resistance				Capillary resistance			
	Göthlin's method		Dalldorf's method		Göthlin's method		Dalldorf's method		Göthlin's method		Dalldorf's method		Göthlin's method		Dalldorf's method	
	85 mm.	50 mm.	cm.	Range for week, cm.	35 mm.	50 mm.	cm.	Range for week, cm.	35 mm.	50 mm.	cm.	Range for week, cm.	85 mm.	50 mm.	cm.	Range for week, cm.
Usual diet	0	1	30	20-30	99	0	3	40	63							
Usual diet	0	5	30	25-30	66	0	4	35	25-35	37						
Usual diet	0	3	35	20-35	84	1	3	45	30-45	91						
Usual diet	0	2	54	20-54	65	5	8	40	30-45	89	0	1	25	0	2	....
Basal diet																
2nd day	2	10	15	15-20	15	0	4	30	30-35	21	2	4	15	0	1	20
9th day	0	10	15	15-25	12	2	5	25	20-40	14	0	3	15	0	5	10
16th day	2	8	15	15-20	11	0	3	40	25-40	12	0	2	20	0	5	15
23rd day	0	8	15	15-20	12	0	7	25	25-35	10	0	1	10	0	4	15
30th day	1	4	20	15-20	7	0	3	30	25-40	6	0	3	15	0	9	10
Recovery period																
7th day	0	9	20	10-25	52	6	12	25	25-40	13	1	1	15	0	3	15
14th day	3	5	20	10-25	149	4	8	30	25-40	110	4	6	15	0	6	15
Usual diet	1	1	20	20-30	70	2	9	30	30-35	21	1	1	15	0	4	15
Usual diet	5	5	15	....	139	0	1	25	25-35	20	2	3	15	0	5	15
Usual diet	1	4	20	....	105	1	3	40	25-40	14	1	3	20	0	4	10
Usual diet	1	7	25	....	135	1	3	30	30-35	15	1	2	15	0	0	20
Usual diet	0	4	20	15-25	98	0	5	25	25-35	15	0	0	15	0	1	6
Usual diet	2	8	20	....	159	1	8	30	....	24	1	1	15	0	3	20

During the period on the basal diet and the recovery period, the capillary resistance tests and determinations of urinary excretion of ascorbic acid were made on the same day; on the usual diet, determinations were sometimes 2 or 3 days apart. The readings which are in italics are subnormal according to Göthlin's standard.

noted. For instance, on the normal diet just before the experiment when P.O. showed five petechiae at 35 mm. and eight petechiae at 50 mm. pressure by Göthlin's method, the reading by Dalldorf's technic was 40 cm. On another occasion, when this subject had only 1 minute hemorrhage when tested by Göthlin's method at 50 mm. pressure, the reading by Dalldorf's technic was 25 cm.

It is evident that the data on capillary resistance do not show a correlation with urinary excretion of vitamin C.

In these experiments, it cannot be said that the determination of capillary resistance by either method gave an adequate indication of the state of nutrition in regard to vitamin C.

Variations in capillary resistance could not be correlated with the menstrual period.

#### SUMMARY

In four cases, the amount of vitamin C necessary to restore the tissues to saturation after a month on a diet very low in vitamin C, ranged from 2200 to 2800 mg., when administered in daily doses of 200 mg.

It is suggested that the difference between intake and excretion up to the point of saturation following prolonged deprivation of vitamin C may afford some indication of the maximum vitamin C reserve. On this basis tissue reserves at saturation appear to be of the order of 2500 to 3000 mg.

In these experiments, capillary resistance did not give an adequate indication of the state of nutrition with respect to vitamin C.



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## A QUANTITATIVE METHOD FOR THE ASSAY OF VITAMIN D WITH CHICKENS

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TWO FIGURES

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The response of chickens, in comparison with the response of rats to a given antirachitic agent, is the principal means of investigating the multiple nature of vitamin D. This was the means used in distinguishing the vitamin D of irradiated ergosterol from that of cod liver oil, of irradiated cholesterol from irradiated ergosterol, and of tuna liver oil from cod liver oil (reviewed by Bills, '35). As increasingly fine distinctions come to be made, improved assay procedure is required.

Heretofore the best available methods for assaying an antirachitic agent with chickens consisted essentially in varying the dosage until a level was found, the effect of which more or less matched that of some standard product. Perfect matching was a matter of rare chance, hardly more significant statistically than the proximate matching obtained with many animals in gradations of the dosage. In the end, the potency of the test substance could be expressed only on a not-more-than or not-less-than basis, subject to an unknown and undeterminable probable error.

It is to be noted that this general type of vitamin assay procedure is still widely used. It is the method, with rats as the test animal, which was recently made official in U.S.P. XI ('35). Quantitative methods differ from it in providing means, such as a graph, whereby a) the response of the test

group can be translated into unitage without the necessity of closely matching the standard response, and b) the probable error of the assay can be estimated. Such methods for the assay of vitamin D with rats have been the subject of detailed studies by several investigators, particularly Bills, Honeywell, Wirick and Nussmeier ('31), Bourdillon, Bruce, Fischmann and Webster ('31), Dyer ('31), Bourdillon and Bruce ('32) and Coward and Key ('33).

#### OUTLINE OF METHOD

A colony of single comb white Leghorn fowls is maintained on a normal ration containing, at all seasons, 200 international units of cod liver oil vitamin D per 100 gm. of feed.<sup>1</sup> Eggs from these hens are selected for hatching. All eggs of imperfect shell or abnormal shape and all which weigh less than 55 gm. the day they are laid, are discarded. Newly hatched chicks are removed to brooders which are heated and illuminated with incandescent lamps of ruby glass. No food is given the chicks before the third day of life, i.e., chicks hatched on Monday are first fed on Wednesday. From the third day of life and into the seventeenth day of life all chicks are fed our rickets-producing diet M, and given tap water to drink.

#### *Diet M*

Ground yellow maize	56
Wheat bran	10
Linseed oil meal	10
Wheat gluten	10
Skimmilk powder <sup>2</sup>	9
Calcium carbonate	2
Sodium chloride	1
Vegetable oil (vehicle for vitamin D supplement)	2
<i>Analysis</i> , protein 21.8, Ca 1.0, P 0.5. Ca: P = 2:1,	

The 2 weeks on diet M is a fore-period during which diseased chicks, weaklings and runts either die or are thrown out. The

<sup>1</sup> An appreciable amount of vitamin D is transmitted from hen to chick, according to Murphy, Hunter and Knandel ('36) and Bethke, Record, Wilder and Kick ('36). Ample vitamin D in the feed should minimize variations in the amount transmitted.

<sup>2</sup> Beware of low grade skimmilk powders made from neutralized milk. The lime used in such products is a possible source of serious error in rickets-producing rations, according to Scott, Hughes and Loy ('32).

remaining strong chicks almost without exception survive the subsequent 4 weeks test period. The fore-period serves also another purpose: It brings the chicks to a state approaching rickets, so that in the test period to follow they will develop severe rickets or become normal birds or show any in-between condition, depending upon the amount of vitamin D now added to the basal diet.

Our method is designed particularly for the assay of oils and powdered milks. When the substance to be assayed is an oil or an oil-soluble material, it is incorporated in the 2% of vegetable oil of diet M. We use maize oil or cottonseed oil, but probably any other edible, vitamin D-free vegetable oil could be substituted. When a vitamin D-bearing powdered milk is to be assayed, it is substituted for the skimmilk and oil of diet M. Thus with either class of test substance, the vitamin supplement is added without any essential change in the composition of the basal ration.

At the end of the fore-period, in the seventeenth day of life, the chicks are transferred to assay cages. Five chicks are placed in each cage, the floor dimensions of which are  $50 \times 55$  cm. In such close quarters the birds are restricted as to exercise; this may or may not be a factor of importance. The cages are provided with screen bottoms. The room is dimly lighted and maintained at about  $27^{\circ}$ . Ten chickens constitute the standard test group. They are given diet M containing the supplement of vitamin D for 28 days. Both the diet and tap water are supplied ad libitum. The supplemented diet is made up fresh each week and kept cold when not in use.

During the twenty-eighth day of the assay period (forty-fifth day of life) the chickens are killed. One femur from each bird is immediately dissected out, and placed in boiling water for 1 minute. It is then scraped free of adherent tissue, care being taken to avoid removal of the still cartilaginous epiphyses (Bethke and Record, '34). Skillful bone cleaning is important. In rickety bones the cortex is so easily abraded that the scalpel used should be somewhat dull. The bones

are broken in three pieces, and extracted in a Soxhlet apparatus for 12 hours with alcohol and 12 hours with ether. The extracted bones are dried to constant weight at 100°, then ashed to whiteness in an electric muffle furnace, the temperature of which is gradually raised to 700°  $\pm$  20°.

TABLE 1  
*Interpretation of ash findings in assays with ten chickens*

I	II	III		IV		V	
Ash	Probable error (b)	I.U. of vitamin D per 100 gm. of ration		Probable error (d)		Probable error (e)	
		C.I. oil	Irr. erg.	Cod liver oil	Irr. ergosterol	Cod liver oil	Irr. ergosterol
%							
36.0	$\pm 0.41$	2.0	40	+ 22; - 43	+ 41; - 44	+ 23; - 43	+ 41; - 44
37.0	0.43	3.1	80	15; 15	22; 22	16; 16	22; 22
38.0	0.45	4.2	120	12; 12	15; 15	13; 13	16; 16
39.0	0.46	5.3	160	10; 10	12; 12	11; 11	13; 13
40.0	0.47	6.4	200	8; 8	9; 9	10; 10	11; 11
41.0	0.47	7.4	240	7; 7	10; 8	9; 9	11; 10
42.0	0.47	8.5	293	7; 6	11; 9	9; 8	13; 11
43.0	0.45	9.9	372	8; 7	13; 11	10; 9	15; 12
44.0	0.44	11.8	498	8; 7	16; 13	10; 9	17; 14
44.5	0.42	12.8	593	8; 7	19; 14	9; 9	19; 15
45.0	0.41	14.0	726	8; 7	21; 16	9; 9	22; 17
45.5	0.39	15.2	922	8; 7	25; 18	9; 9	26; 19
46.0	0.37	16.7	1240	8; 7	31; 20	10; 9	32; 21
46.2	0.37	17.4	1420	8; 7	35; 22	10; 9	35; 22
46.4	0.36	18.1	1660	8; 7	40; 23	10; 9	40; 24
46.6	0.35	18.8	1970	8; 7	47; 26	10; 9	47; 26
46.8	0.34	19.7	2410	8; 7	56; 28	10; 9	56; 29
47.0	0.33	20.6	3060	9; 7	70; 31	10; 9	71; 32
47.2	0.32	21.6	4100	9; 7	97; 36	11; 9	97; 36
47.4	0.31	22.8	5980	10; 8	42	12; 10	43
47.6	0.30	24.2	10000	12; 8	51	13; 10	52
47.8	0.29	25.9		15; 9		16; 11	
48.0	0.28	28.3		50; 11		51; 12	

The average percentage of ash in the fat-free, moisture-free femurs of the ten birds comprising the assay group is located in column I of table 1. The corresponding number of international units (I.U.) of vitamin D per 100 gm. of ration is read from column III. When the assay has been conducted without the use of a positive control group the probable error,

$PE_e$ , expressed as per cent of the number of units, is read directly from column V. When a positive control group has been used (e.g., a standard reference oil or any other substance, in terms of which the test substance is being assayed), the probable error,  $PE_t$ , is calculated by substituting in formula 7 the value of  $PE_d$  taken from column IV. When the number of chickens is other than the regular number, ten, the calculations of  $PE_e$  or  $PE_t$  are computed from  $PE_b$  in column II, by substituting it in formula 8 and following through as described under 'Probable Error.'

#### EXPERIMENTAL

The single comb white Leghorn was chosen as the test bird because it is the variety of fowl most widely available and already the most commonly used in experimental studies.

The basal ration, diet M, was planned so that, except for the lack of vitamin D, it is a normal diet for young chicks. Constancy in its P content is favored by the multiplicity of P-bearing ingredients. Most of its Ca is supplied by the added  $CaCO_3$ .

The Ca:P ratio of the diet is 2:1. Ratios between 2:1 and 1:1 are optimal for young chickens, yet, as is well known, chickens develop rickets, no matter how favorable the ratio, unless vitamin D is supplied. Thus an unbalance in Ca:P, essential in rickets-producing diets for rats, is not required for chicks. In fact, it may even be undesirable. Preliminary experiments with several hundred chicks were performed, in which Ca:P was varied from 4:1 to 1:4, and several levels of vitamin D were administered with each ratio. There was no advantage in the abnormal ratios, although they were indeed provocative of bone disorders, so much so that death within the assay period frequently ended the experiment.

The basis for interpreting the findings of an assay was developed from a study of response curves, or ideal series, shown in figure 1. These master curves were obtained by administering graded doses of vitamin D in the form of cod liver oil and of irradiated ergosterol. The cod liver oil was

rendered solely from the livers of the common Atlantic codfish, *Gadus morrhua*. It contained 77 I.U. ( $\pm 5.6\%$ ) of vitamin D per gram and about 1300 I.U. of vitamin A per gram. These values are within the ordinary limits of variation for pure

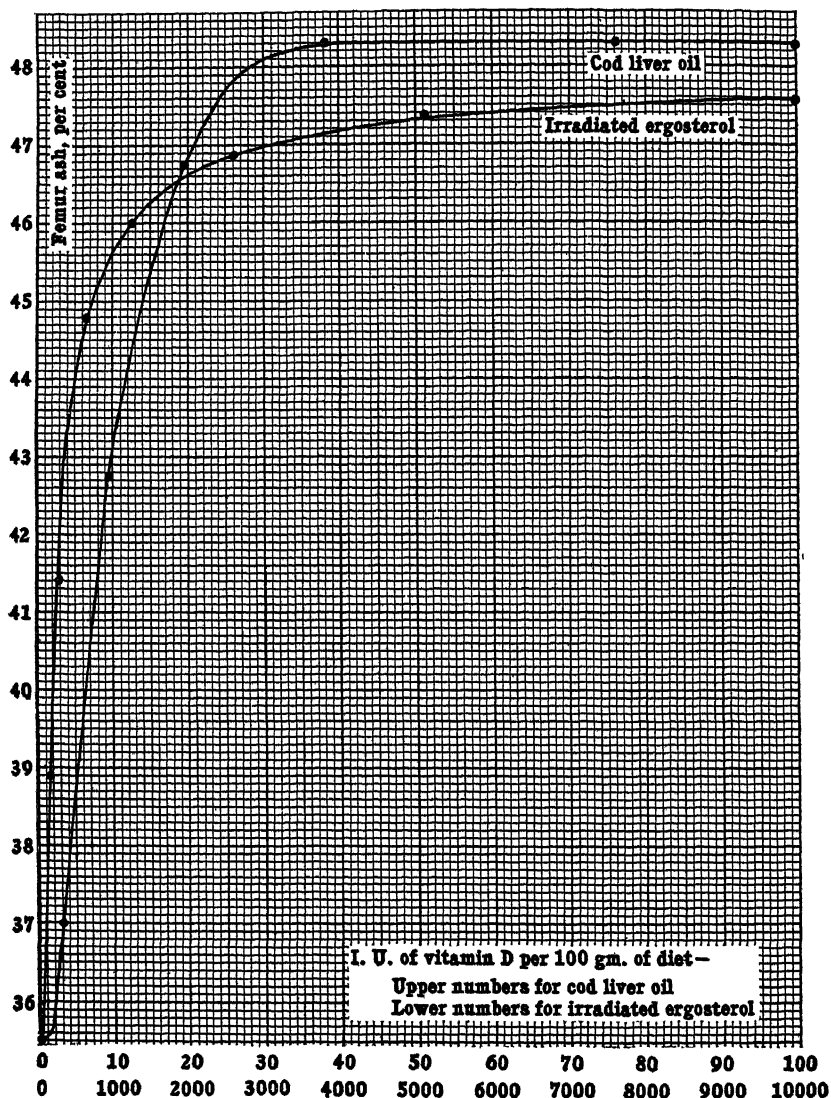


Fig. 1 Master curves of response.



cod liver oil, the average potency of which is 100 I.U. of vitamin D and 1000 I.U. of vitamin A per gram. They are somewhat lower than the values for many commercial specimens of 'cod liver oil,' the superior potency of which is due to the legally permitted admixture of liver oils from other species of the family Gadidae. The irradiated ergosterol was prepared as described by Bills, McDonald, BeMiller, Steel and Nussmeier ('31). It was dissolved in maize oil to give a stock solution containing 10,000 I.U. ( $\pm 6.1\%$ ) of vitamin D per gram.

Dilutions of the cod liver oil and of the irradiated ergosterol stock solution were prepared in maize oil so that the desired unitage per 100 gm. of diet was supplied. Besides the zero level, 6 levels of the vitamin as cod liver oil and 7 as irradiated ergosterol, each indicated on the curves of figure 1, were triturated with the diet and administered to the chicks. The number of chicks used to establish each of the points determining the master curves was, of course, larger than the number for an ordinary assay. From twenty-five to fifty chicks (total, 519; average, 37) were used for each point, the larger groups being used at the ends and bends of the curves. The chickens were taken over the greater part of a year, and those of any given hatch were distributed over several dosage levels. Distribution as to sex was fortuitous.

On the curves of figure 1, the determining points were established by plotting the number of units administered per 100 gm. of diet as abscissas; the average percentages of femur ash corresponding to the unitage as ordinates. The general shape of the curves was not unexpected. Beginning at a threshold corresponding to the first definite betterment of the ricketic bone, and continuing halfway toward normal bone, the relation of units to ash is essentially linear. Thereafter, an ever increasing number of units of vitamin D are required to effect a degree of betterment in bone. Such, perhaps, is the course of most physiological responses in the direction of satiety.

Unless drawn on a very large scale, the curves in figure 1 cannot be read with sufficient accuracy for the interpretation of the ash findings in an assay. The curve readings in column III of table 1 were therefore not taken from the curves directly, but were calculated from equations of the curves which were determined for us by Mr. Delbert Deisinger.

A striking feature of the response curves for cod liver oil and irradiated ergosterol is their essentially different shapes. They cannot be made to coincide, by any magnification or contraction of abscissas. As drawn, they appear to intersect, but this appearance is an artifact due to the use of two series of abscissas for convenience in graphic portrayal. The artifact, however, draws attention to the great difference in the responses induced by these two forms of vitamin D. Where the curves 'intersect,' i.e., where approximately 46.5% of femur ash is obtained, the effectiveness of cod liver oil, rat unit for rat unit, is exactly 100 times as great as that of irradiated ergosterol for the chicken. Below this point, the difference in effectiveness gradually decreases, e.g., at 40.0% ash it is only thirty-one times. Above this point, the difference becomes great, e.g., at 47.3% ash it is 220 times. Thus we are able to explain the hitherto perplexing fact, discussed by Bethke, Record and Kennard ('33), that different investigators, comparing the efficacy of cod liver oil with that of irradiated ergosterol (or substances containing it), rat unit for rat unit on chickens, came to widely different conclusions. The two agents are simply not comparable in broad terms. Their relative effectiveness is determined by a number of factors, according to King, Hull and Hall ('33) and Bills, Massengale, McDonald and Wirick ('35). It now appears that the most important factor is the dosage at which the vitamin is given.

Under the conditions of our experiments, it is reasonable to regard a femur ash of between 46 and 47% as normal (compare fig. 1). Thus qualified, the effectiveness of the vitamin D of cod liver oil is 100 times that of irradiated ergosterol, rat unit for rat unit on chickens. This is the value originally

established by Massengale and Nussmeier ('30) under somewhat different experimental conditions. The amount of cod liver oil vitamin D required to give 'normal' bone with the 'normal' diet used is 18 I.U. per 100 gm. of ration.

#### PROBABLE ERROR

Estimation of the probable error in any given assay by means of table 1 is manifestly simple, but the computations by which table 1 was developed involve a chain of considerations. Several ways of expressing error were considered, but the method chosen as the most useful was that which expresses it as percentage of the number of units found by the assay.

One determinant of the probable error is the deviation to which the femur ash percentage of the ten chicks comprising the assay group is subject. Since the data from any individual group without reference to other groups are not very significant statistically, it seemed rational, as well as exceedingly convenient, to determine the average deviation, once and for all, by the analysis of many groups. This was done with the data from 160 groups of ten chicks each, including most of the chicks used in the ideal series. The average deviation in femur ash percentage was separately reckoned for each of the 160 groups. Still separately, these average deviations were converted into the probable errors of the groups by multiplying by 0.8453 (Peter's factor) and dividing by the square root of one less than the number of chickens in each group,

$$\text{Formula 1: } PE_a = \frac{0.8453 \times AD}{\sqrt{10-1}} = \frac{0.8453 \times AD}{3}$$

Thus we obtained 160 evaluations of  $PE_a$ , the probable error in ash percentage, which were to be expressed later as probable error in number of units,  $PE_u$ , and in per cent of units,  $PE_p$ . Separate computations showed no significant differences in  $PE_a$  when cod liver oil, other fish oils, or irradiated ergosterol supplied the calcifying agent.

At this point, a second determinant of error was found in the fact that the deviations in ash varied with the amount of ash. Bones of nearly normal ash content varied less than the

softer bones, perhaps as the result of their proximity to the limit of calcification or perhaps because they were less susceptible to mutilation in the hands of the assayist. The most variable bones were those partially calcified. Whatever the reasons for the variations, the fact precluded the averaging of the 160 values of  $PE_a$  and necessitated a graphic interpretation of them. The probable errors in ash were located on coordinate paper as shown in figure 2. Here the trend is clearly discernible despite the irregularities in the ordinates, which, as a matter of fact, are exaggerated ten times with

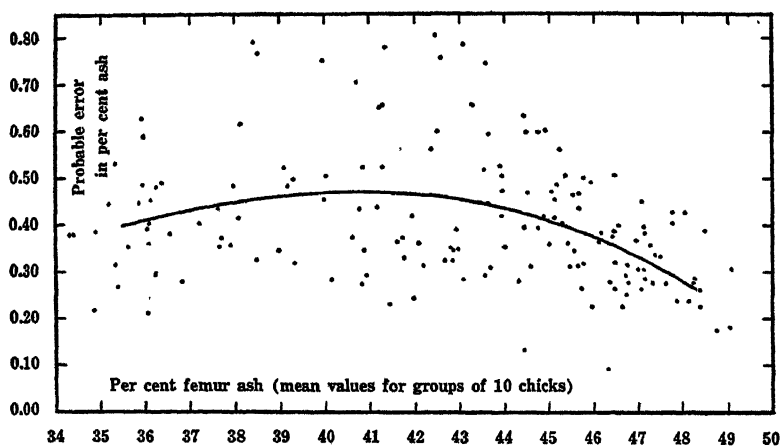


Fig. 2 Error in ash as a function of the amount of ash. (The vertical scatter of points is exaggerated ten times, for convenience in illustration).

respect to the abscissas as a convenience in making the drawing. No points were thrown out, however extreme the error which they represented. The points were arithmetically consolidated into ten clusters of sixteen points each, taken in order of ash percentage. From the ten new points representing the cluster centers, the curve in figure 2 was constructed. The curve broadens our knowledge of the errors, previously restricted to 160 instances, so that we can now state the probable error in ash which is associated with any degree of calcification in the typical assay with ten chickens. In distinction from  $PE_a$ , we designate the generalized curve readings as  $PE_b$  (column II in table 1).

The third determinant of error is the varying relation between ash produced and unitage required to produce it. This is represented in the slopes of the curves in figure 1, from which one notes that a given amount of difference in ash affects the number of units less where the curves are steep than where they have flattened out. Error in ash was translated into error in units,  $PE_c$ , in accordance with the equations,

$$\text{Formula 2: } PE_{c, \text{ plus}} = U' - U, \text{ and}$$

$$\text{Formula 3: } PE_{c, \text{ minus}} = U - U'', \text{ where}$$

$PE_c$  is the probable error, plus or minus, to which the number of I.U. of vitamin D per 100 gm. of ration, corresponding to the found percentage of femur ash in ten chicks, is subject;  $U$  is the number of I.U. of vitamin D required in 100 gm. of ration to give the found percentage of femur ash;  $U'$  is the number of I.U. of vitamin D required in 100 gm. of ration to give a percentage of femur ash equal to the found percentage plus its probable error,  $PE_b$ ; and,  $U''$  is the number of I.U. of vitamin D required in 100 gm. of ration to give a percentage of femur ash equal to the found percentage minus its probable error,  $PE_b$ .

The values for  $PE_c$  were read from an enlargement of the curve in figure 2, and the values for  $U$ ,  $U'$  and  $U''$  were calculated from the equations of the curves of figure 1.

The fourth determinant of error, when error is re-expressed percentagewise, is the number of units per 100 gm. of ration. Since  $U$ , in the necessary calculations, is the divisor, it is apparent that error in per cent, designated  $PE_d$ , is greatest when  $U$  is smallest. The equations are obvious:

$$\text{Formula 4: } PE_{d, \text{ plus}} = \frac{PE_{c, \text{ plus}} \times 100}{U} \text{ and}$$

$$\text{Formula 5: } PE_{d, \text{ minus}} = \frac{PE_{c, \text{ minus}} \times 100}{U}$$

In table 1 the values for  $PE_d$  represent the error which is inherent in the method of comparing a group of ten chicks with an errorless ideal series, no controls being used. But a fifth determinant of error enters here, in the fact that the cod liver oil and the irradiated ergosterol solution with which the master curves were established, were themselves subject

to an assay error,  $PE_s$ . They had been standardized with rats against the international reference solution of irradiated ergosterol issued by the Health Organisation of the League of Nations ('31). The procedure was that described in our critique of the line test (Bills et al., '31), with certain minor improvements. The probable error of the standard,  $PE_s$ , was  $\pm 5.6\%$  with the cod liver oil, and  $\pm 6.1\%$  with the irradiated ergosterol. Taking this into consideration, we finally arrive at the value for the probable error,  $PE_e$ , expressed in per cent of the number of units found by an assay with ten chickens, without any controls other than those represented in the master curves,

$$\text{Formula 6: } PE_e = \sqrt{PE_d^2 + PE_s^2}$$

Values of  $PE_e$  are tabulated in column V of table 1.

It is sometimes desired to assay one vitamin D source in terms of another, rather than in terms of the master curves. For instance, one may wish to assay a sample of cod liver oil in terms of an official reference oil.<sup>3</sup> In such assays two groups of ten chickens each must be used, one for each of the substances. The error,  $PE_s$ , is eliminated, but another error is introduced by the additional assay group. The probable error of the end result,  $PE_r$ , is found by a modification of formula 6, namely,

$$\text{Formula 7: } PE_r = \sqrt{PE_{d1}^2 + PE_{d2}^2}$$

where  $PE_{d1}$  and  $PE_{d2}$  are the  $PE_d$  values of the two groups of chicks. Values of  $PE_r$  are not given in the tables, since they must be reckoned separately for each problem that arises.

To illustrate the use of formula 7, suppose that two cod liver oils, X and Y, were assayed with ten chickens each. Oil X showed 160 I.U., and oil Y 80 I.U. of vitamin D per gram.

<sup>3</sup> Although the U.S.P. XI ('35) unit and the international unit are by definition identical, it does not follow that the declared potency of the U.S.P. reference cod liver oil is free of error in terms of the international standard. Our experience has been that the current (first) issue of the U.S.P. reference oil, which nominally contains 95 U.S.P. units of vitamin D per gram, actually shows but  $80 \pm 2$  I.U. of vitamin D per gram when assayed most carefully with rats against the international standard. It is emphasized that our master curves were based upon the international standard, not upon the reference oil.

Each value, of course, was subject to error. For most purposes the computations would end here, but if it were desired to express X in terms of Y, one would divide 160 by 80, finding that X is two times as potent as Y. To determine the probable error of this quotient, one would make a special computation from the  $PE_a$  values in column IV instead of taking the  $PE_a$  values in column V. Let us say that the  $PE_a$  values of the two assays were x and y, respectively. Substituting in formula 7,

$$PE_x = \sqrt{x^2 + y^2}$$

which is the probable error of oil X in terms of oil Y. This value is usually somewhat greater than the larger of the  $PE_a$  values of the two assays. Even when oil Y is an official standard, such as the U.S.P. reference cod liver oil (which by definition contains a certain number of U.S.P. units<sup>3</sup>), the procedure remains the same for the computation of error. After the quotient of X over Y has been determined, one multiplies this quotient by the officially defined potency of Y, to express the potency of X in terms of the units used in the official definition of Y.

*The sex error.* In the line test with rachitic rats it has been reported that males deposit slightly more calcium than females in response to semi-curative doses of vitamin D (Bills et al., '31; Bourdillon et al., '31; and Coward and Key, '33). The difference is so small, and so interwoven with other variables that investigators agree it is of no practical significance. In studies with normal chickens it has been found that male tibias contain slightly less ash than female tibias. From the work of Holmes, Pigott and Moore ('32) and Schroeder ('33) it appears that the ratio of tibia ash percentage in males to that in females varies with the age of the chicks as follows:

21 days — M: F =	101.1 : 100	(Holmes et al., '32)
42 days — M: F =	97.7 : 100	(Holmes et al., '32)
63 days — M: F =	96.8 : 100	(Holmes et al., '32)
70 days — M: F =	96.4 : 100	(Schroeder, '33)

If a similar relation holds for femur ash, one can, by interpolation, estimate that for 45-day chicks the M: F ash ratio is about 97.5:100. On this basis, an assay of cod liver oil con-

ducted with cockerels only would figure out 8% fewer units than one with both sexes, and an assay with pullets only would figure out 7% more units than one with both sexes. (It is assumed that the chicks with which the master curves were constructed had a sex ratio of 1:1). Similarly, if the substance assayed were irradiated ergosterol, the differences would amount to 9% and 12% for cockerels and pullets, respectively. These calculations are based on a found femur ash of 41.4%, wherewith the associated probable error is approximately minimal.

It should be borne in mind that all calculations of the probable error in our method include the error due to sex. It remains now to be determined whether or not reduction of the total probable error by correction for sex error is feasible. It is, of course, impossible for the average worker to ascertain the sex of chickens at the beginning of an assay, so that the correction, if made at all, would have to be made at the end.

Assuming that the sex ratio of chickens is unity, one learns by the factorial equation,

$${}_nC_r = \frac{n!}{r!(n-r)!}$$

that the chances of any assembly of ten chicks being either all cockerels or all pullets are only two in 1024. But the chances are 912 in 1024 (about 89%) that the sex ratio will be one or another of the following, 7:3, 6:4, 5:5, 4:6 or 3:7. Here the sex error is inconsequential. There remain 110 chances in 1024 (about 11%) that a ratio of 2:8, 8:2, 1:9 or 9:1 will obtain, in which the sex error is small, but appreciable.

The importance of sex, small as it thus appears, is even smaller, for it has been shown by Lachat ('34) and Lachat and Halvorson ('36 a, b) that in rachitic chicks, unlike chicks that have received adequate or nearly adequate amounts of vitamin D, the bone ash difference associated with sex is insignificant. It therefore would seem that in our procedure, correction for sex error is not worth while.

*Use of controls.* Were it possible to have an assay technic flawless in every detail, controls would merely confirm the master curves upon which the technic was based. Under



practical conditions, negative controls have little value, but positive controls are useful. The latter serve to assure that gross errors, such as incorrect compounding of basal diet, have not entered the work. They also compensate for periodic errors, such as the seasonal variation in bone ash which Lachat ('34) and Lachat and Halvorson ('36 a, b) have observed in rachitic chickens. To some extent, however, the error due to seasonal variation is already included in the calculations of probable error in our method.

When the positive controls are run merely to assure that the response of the test birds is not materially out of line with the master curves, i.e., when error is to be estimated as  $PE_o$  of column V in table 1, it is well to remember that error is as likely to occur in the control group as in the test group, and that deviations in the control group average from the master curves of less than three times the probable error need not be considered significant unless they are consistently repeated. When the positive controls are run as a standard reference oil, in terms of which the potency of the test substance is to be calculated, i.e., when error is to be computed as  $PE_t$  via  $PE_a$  of column IV in table 1, it should be recognized that control group error is not thereby eliminated, but is merely hidden.

*Use of a number of chickens other than ten.* All data on error in table 1 are based on the use of ten chickens in each assay group. It is possible, however, to adjust the calculation of error to numbers of chickens other than ten. In so far as error is determined by the number of birds, it varies inversely with the square root of one less than the number, but since there are other determinants of error than number of birds, one cannot apply this inverse relationship to the final calculations of  $PE_o$  or  $PE_t$ . One must introduce the correction in  $PE_o$ , and thence carry it through each successive formula to the final computations. It is for this purpose that values of  $PE_o$  are given in column II of table 1. To obtain the value of  $PE_o$  adjusted for  $n$  birds, one solves—

$$\text{Formula 8: } PE_{bn} = \frac{\sqrt{10-1} \times PE_o}{\sqrt{n-1}} = \frac{3 \times PE_o}{\sqrt{n-1}}$$

The corrected values thus obtained are not wholly valid, especially for small groups, because certain errors, such as the error due to sex, increase disproportionately when the number of animals is substantially less than ten.

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#### SUMMARY

1. A quantitative method is described for the assay of vitamin D with chickens. It is based on the determination of femur ash, and the interpretation of the ash percentage by means of either of two response curves, one for cod liver oil and the other for irradiated ergosterol.

2. The method has been analyzed in regard to probable error, and means provided for the easy estimation of error, with any given number of chickens, in both direct assays and in assays of one substance in terms of another. Under the most favorable conditions the probable error of an assay of irradiated ergosterol with ten chickens is as little as 9% of the unitage found. A slightly smaller error is experienced in assays of cod liver oil.

3. With the diet used, which had  $\text{Ca}:\text{P} = 2:1$  (optimal for young chicks), good calcification resulted from the addition of 18 I.U. of vitamin D, as cod liver oil, per 100 gm. of ration.

4. Explanation of conflicting reports on the relative effectiveness, rat unit for rat unit, of cod liver oil and irradiated ergosterol for chickens, was found in the fact that these sources are not broadly comparable. Their relative efficacy fluctuates with the degree of calcification, the difference being small at low calcifications, 100 times at 'normal' calcification, and very great in the production of extra-hard bone.

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## SUSCEPTIBILITY OF DIFFERENT STRAINS OF RATS TO NUTRITIONAL CATARACT<sup>1</sup>

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ONE FIGURE

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In earlier papers (Mitchell, '35; Mitchell and Dodge, '35) the author reported the occurrence of cataract in white rats fed on lactose and galactose. The incidence of mature bilateral cataract in this first series of experiments in which 70, 50 and 30% lactose rations were fed was 69, 27 and 0%, respectively. The incidence was 100% on the 35% galactose ration. All rats in these experiments were from the Battle Creek colony which originated 12 years previously from a cross between Wistar and Yale strains and were between 24 and 30 days of age when started on the experimental rations.

Yudkin and Arnold ('35) using rats from the Yale colony obtained less than a 50% incidence of cataract on the 70% lactose ration. A limited number of rats from the University of Wisconsin colony fed on the 70% lactose ration developed mature cataract at a slower rate than animals from our own colony. A personal communication from Michigan State College informed us that in their colony a few cataracts developed when high levels of lactose were fed. The rations were not exactly like those in our work nor was the exact incidence of cataract recorded. Such casual observations,

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however, suggested a possible variation in susceptibility to cataract among different strains of rats.

When the cataract research project was reestablished in a new institution rats from the local colony (M.S.C.) were used in some preliminary experiments. They proved exceedingly resistant to lens changes of the more severe type. Rats from a pure Wistar strain (W.) were next used on the 70% lactose ration and cataracts again failed to develop in the usual time. Finally rats from the Battle Creek colony (B.C.) were again tested and found to be equally if not slightly more susceptible to cataract than in our previous series of experiments with the same strain. This striking difference in breeds which retarded progress in our main research program nevertheless prompted us to make further observations on the response of different strains to the same rations. More animals from each of the albino strains mentioned were fed the two cataract producing rations. Later a group of 'pied' rats (J.H.) from the biochemistry department of the Johns Hopkins University were fed the galactose diet.

#### EXPERIMENTAL

*Procedure.* Growth, food intake, abnormal intestinal conditions, early lens changes and incidence of mature cataract were recorded regularly for all animals. Table 1 summarizes the significant findings on the three strains of rats fed on the 70% lactose ration and the four strains fed on the 35% galactose ration. The number of rats from the B.C. strain was naturally much greater than from any of the others since it proved expedient to use rats from this colony for all subsequent observations on cataract. The possibility of a sex difference in susceptibility to cataract was also considered, for which reason the data on males and females were recorded separately.

*Susceptibility to cataract.* The incidence of cataract in the various breeds and the time required for development of complete opacities is graphically told in figure 1. On the 70% lactose ration the high incidence of mature cataract in the

ninety-eight rats of the B.C. strain is the most significant. Advanced lens changes appeared in all of the animals and mature cataract was visible to the naked eye in 82 and 89% respectively of the males and females. It is obvious that the

TABLE 1

*Response of different strains of rats to lactose, galactose and starch rations*

RATION	STRAIN	NUMBER OF RATS	RATE OF GROWTH	INTESTINAL CONDITION	TIME IN DAYS FOR DEVELOPMENT OF MATURE CATARACT			INCIDENCE OF CATARACT PER CENT
					Min.	Max.	Average	
70% lactose	B.C.♂	52	Delayed	Severe	14	80	44	82
	B.C.♀	46	Delayed	Diarrhea	18	104	45	89
	M.S.C.	10	Delayed	Diarrhea	49	63	56	10
	W.	11	Delayed	Distention and diarrhea	97	97	97	9
35% galactose	B.C.♂	34	Normal	Normal	11	26	16	100
	B.C.♀	42	Normal	Normal	12	28	17	100
	M.S.C.	6	Normal	Normal	21	44	32	100
	W.	10	Normal	Normal	21	49	29	60
	J.H.	15	Normal	Normal	16	45	23	83
70% starch	B.C.	55	Normal	Normal	..	..	..	0
	M.S.C.	4	Normal	Normal	..	..	..	0
	W.	4	Normal	Normal	..	..	..	0
	J.H.	4	Normal	Normal	..	..	..	0

*Cataract Development in Different Strains of Rats*

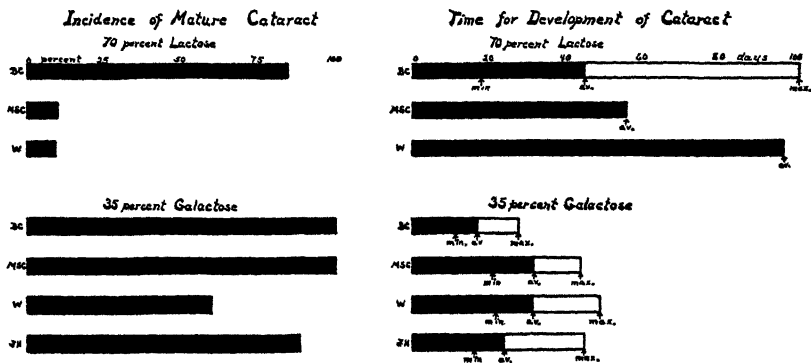


Figure 1

difference between the sexes is insignificant. The longer time recorded for cataract development in the other strains becomes more striking when one notes that only two eyes out of twenty in the M.S.C. strain and two out of twenty-two in the W. strain ever showed complete opacities, although all of the animals from these strains remained on the 70% lactose ration well beyond the time when cataract might be expected to develop.

On the 35% galactose ration the incidence of mature cataract is high in all four strains, 100% in the B.C. and M.S.C. rats. The time required for the development of cataract in the different breeds is particularly interesting. The time is shortest and surprisingly constant for the B.C. rats, the others showing a slower development on the average and a much greater variation in time within a given litter.

*Growth.* There was no apparent difference between strains as regards rate of growth on the two rations. All showed delayed growth on the 70% lactose diet but tended to resume a better rate of growth after a few weeks when a tolerance for this sugar seemed to have developed. All strains grew at an approximately normal rate on the 35% galactose diet for the few weeks during which they remained on this ration. The galactose feeding was usually discontinued as soon as cataract developed, that is within 3 or 4 weeks.

*Intestinal condition.* The severity of intestinal disturbances showed no correlation with cataractous changes. The severe diarrhea resulting from feeding high levels of lactose is familiar to all who have used such rations. There was no exception to the rule for any of the rats used in this study, but the nature of the intestinal disturbance seemed to vary with the strain. A more or less severe diarrhea occurred in all and autopsy usually disclosed a much enlarged cecum filled with a fluid or semi-solid mass. In the Wistar rats the distension was more extreme and largely due to an accumulation of gas. The 'pot bellied' appearance of these rats was conspicuous even to the casual observer. When one recalls that this same strain was peculiarly resistant to lens injury



from this lactose diet, the question naturally arises as to how much of the sugar was actually absorbed into the blood stream. It is regretted that systematic blood sugar studies could not have been made on all animals but it is a promising field for further investigation. In a limited series of determinations made under standard conditions the total blood sugar levels were high, chiefly due to the non-fermentable fraction. Breed difference in this respect has not been investigated. Since completing this study the author has discovered a pertinent comment in a recent paper by Donhoffer ('35) who states that each breed of rat is a law unto itself as regards the rate of absorption of various sugars. Therein may lie the secret of the breed difference in susceptibility to cataract.

There was no apparent diarrhea or other intestinal disturbance in any of the rats on the 35% galactose ration regardless of the strain. The potent cataract producing action of galactose has previously been attributed to the inability of the rat to utilize this sugar as rapidly as it is absorbed from the gut (Mitchell, '35). The suggestion made by Dodge ('35) that there may be a correlation between the severity of intestinal abnormalities observed on the lactose rations and the incidence of cataract is quite definitely contradicted by these findings. The most rapid lens changes occur in rats where the intestinal condition appears to be normal. His conclusions are based upon a far too limited number of data and the use of but one basal diet for the production of cataract.

*Age when started.* The age at which rats are started on the experimental rations is also an important factor in the rate of cataract development as well as in total incidence. Although no difference in strain was noted in this respect all comparisons should be made with the age factor as nearly constant as possible. In our earlier series of experiments a slower development of lens changes was noted when the rats were started on the 70% lactose ration at 60 days of age in contrast to the usual procedure in which the age range was

from 25 to 30 days. In a later series of experiments, rats started at 120 days of age failed completely to develop mature cataract although they showed some early lens changes when observed with the ophthalmoscope. Yudkin and Arnold ('35) noted delayed cataract development in older rats on galactose rations. Young rats do not tolerate an abrupt change from a stock ration to the 70% lactose ration, which necessitates a gradual increase to this level over a period of 3 or 4 days. The 35% galactose ration is well tolerated by rats 25 days old or even younger.

#### CONCLUSIONS

It is quite possible that breed difference in rats used may account for the apparent discrepancy in observations from various laboratories. Bourne and Pyke ('35) reported a lower incidence of cataract due to vitamin G ( $B_2$ ) deficiency than did Langston and Day ('33) and Day, Langston and O'Brien ('31). Although this is another type of nutritional cataract than the one reported in this paper it is possible that the difference in breed of rats and a possible difference in the time of starting on the experimental ration has a bearing upon the results obtained. These variations in the incidence of cataract in different strains and at different ages emphasize the necessity for a consistent experimental procedure, a relatively large number of observations, and a consideration of the susceptibility to cataract of the strain of rats used.

#### SUMMARY

1. Rats from different colonies fed the lactose or galactose cataract producing rations showed variable susceptibility to cataract.
2. The B.C. strain had the highest incidence and the mature cataracts developed in the shortest time of any of the three breeds tested on the 70% lactose ration.
3. The M.S.C. and W. strains showed advanced lens changes but complete opacities developed in only 10 and 9%, respectively, of the rats tested.

4. On the 35% galactose rations the incidence of mature bilateral cataract was 100% in B.C. and M.S.C. strains and slightly less in W. and J.H. strains. The time for cataract to develop was shortest in the B.C. strain.

5. Severity of intestinal disturbances did not correlate with cataractous changes in the lens.

6. Growth was normal on the galactose but slightly retarded on the lactose ration, probably due to the persistent diarrhea in the young rats.

7. Cataract development was more rapid in young rats than in older ones. The age at which animals are started on experimental rations must be kept constant if results are to be consistent.

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# THE DISTRIBUTION OF VITAMIN B<sub>4</sub> IN SOME PLANT AND ANIMAL PRODUCTS

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Studies on vitamin B<sub>4</sub> hitherto reported have been concerned for the most part with concentration of this factor, and very little information as to its distribution has been accumulated. Reader ('29, '30) successfully used yeast as a starting material for the concentration of vitamin B<sub>4</sub>, and a previous report from this laboratory (Keenan et al., '33) described the preparation of concentrates from pork liver, blue grass and alfalfa. Chicks were used as the experimental animals in the assay of these concentrates, and evidence was presented to show that the chick paralysis observed here was a manifestation of the same deficiency as the paralysis in rats described by Reader. Reader ('29, '30) presented two methods of assay, one involving the use of young growing rats and the other depending upon the use of adult rats. However, growing chicks are much more susceptible to vitamin B<sub>4</sub> deficiency than are rats, and hence are of greater value for assay of this factor. Recent improvements in the vitamin B<sub>4</sub>-low ration for chicks, as described in a previous paper (Kline et al., '36), suggested a study of vitamin B<sub>4</sub> distribution.

## EXPERIMENTAL

Day-old White Leghorn chicks were obtained from the department of poultry husbandry for these experiments and housed in 14 inch by 16 inch by 14 inch cages equipped with

<sup>1</sup> Published with the permission of the director of the Agricultural Experiment Station.

wire screen floors. The basal ration used in these assays was ration 452, which has the following composition:

Dextrin	64
Reprecipitated casein	18
Salts 1	5
Brewer's yeast	2
Autoclaved liver residue	2
Liver extract	2
Water extracted lung	5
Cod liver oil	2

TABLE 1  
*Vitamin B<sub>1</sub> content of seeds and seed products*

SUPPLEMENT TO RATION 452	NUMBER OF CHICKS	AVERAGE WEIGHT 3 WEEKS	AVERAGE WEIGHT 6 WEEKS	ONSET OF PARALYSIS	NUMBER WITH DEFICIENCY	PER CENT PARALYSIS	DEATHS PRIOR TO 6 WEEKS
None	29	77	...	3	29	100	27
20% Crisco	3	80	134	..	0	0	0
32% Wisconsin 1933 yellow corn	3	106	270	3	2	66	0
32% Wisconsin 1934 yellow corn	3	82	230	3	2	66	0
32% Wisconsin 1935 yellow corn	3	90	287	3	2	66	0
40% Wisconsin 1935 yellow corn	3	148	423	..	0	0	0
24% Texas 1935 yellow corn	4	103	295	2	1	25	1
32% Texas 1935 yellow corn	4	103	315	2	2	50	2
40% Texas 1935 yellow corn	3	152	382	..	0	0	0
24% white corn	4	95	180	3	1	25	1
32% white corn	3	108	285	..	0	0	0
16% wheat	3	90	212	2	3	100	0
24% wheat	3	87	177	4	3	100	0
32% wheat	6	115	320	5	4	66	0
40% wheat	3	135	323	..	0	0	0
40% autoclaved wheat	3	115	320	3	2	66	1
24% hulled oats	4	115	358	3-5	3	75	0
32% hulled oats	4	91	282	..	0	0	0
10% wheat germ	4	75	243	6	1	25	1
15% wheat germ	12	137	350	..	0	0	0
20% wheat germ	3	152	450	..	0	0	0
5% peanuts	6	83	...	3	6	100	6
10% peanuts	6	108	271	4	6	100	0
15% peanuts	20	130	355	..	0	0	0
10% NaCN dried grass	6	132	285	..	0	0	0
15% defatted wheat germ	4	136	...	3	4	100	4
20% defatted wheat germ	3	143	325	..	0	0	0

A detailed description of the constituents of this ration is given in a previous paper (Kline et al., '36). The various levels of the materials to be assayed were substituted in this ration for equal amounts of dextrin.

TABLE 2  
*Vitamin B<sub>4</sub> assay of animal products*

SUPPLEMENT TO RATION 452	NUMBER OF CHICKS	AVERAGE WEIGHT 5 WEEKS	AVERAGE WEIGHT 6 WEEKS	ONSET OF PARALYSIS	NUMBER WITH DEFICIENCY	PER CENT PARALYSIS	DEATHS PRIOR TO 6 WEEKS
12% dried egg yolk	3	130	297	<i>weeks</i> 3	1	33	1
10% vacuum dried pork liver	4	66	238	..	0	0	0
18% vacuum dried pork liver	4	108	298	4	2	50	1
24% vacuum dried pork liver	3	175	452	..	0	0	0
15% dried pork brain	3	130	305	..	0	0	0
15% NaCN dried pork brain	3	147	360	..	0	0	0
10% NaCN dried pork brain	4	97	242	4	1	25	1
15% skim milk powder	3	82	...	3	3	100	3
10% pork kidney (dried)	3	102	248	6	2	66	0
15% pork kidney (dried)	3	130	340	..	0	0	0
15% spinal cord	3	75	...	4	1	33	3

TABLE 3  
*Vitamin B<sub>4</sub> potency of plant and animal products*

	Minimum protective level, per cent		Minimum protective level, per cent
NaCN dried grass	< 10	White corn	32
Peanuts	15	Hulled oats	32
Wheat germ	15	Wheat	40
Pork brain	15	Yellow corn	40
Pork kidney	15	Egg yolk	> 12
Crisco	< 20	Skim milk powder	> 15
Defatted wheat germ	20	Spinal cord	> 15
Pork liver	24		

Tables 1 and 2 give the results of assays of plant and animal products, respectively. The grains used were obtained locally except for the sample of Texas yellow corn which was furnished by Dr. A. R. Kemmerer of the Texas Agricultural Experiment Station. The wheat germ and defatted wheat germ were commercial products sold for human consumption under the trade

names 'Embo' and 'Vio Bin,' respectively. The peanuts were unroasted, but dried at 50°C. The materials designated 'NaCN dried' were dried at room temperature after first adding sufficient NaCN solution to bring the concentration to M/1000. The vacuum dried pork liver and spinal cord were obtained from Dr. David Klein of the Wilson Laboratories, Chicago, and the other animal products were obtained from a local packing plant and dried at 50°C. unless otherwise indicated.

Table 3 gives the minimum protective levels of the materials assayed.

#### DISCUSSION

The data here presented show the grains to be relatively poor sources of vitamin B<sub>4</sub>. The superiority of white corn and hulled oats to yellow corn and wheat is of interest, as is also the superiority of wheat germ to whole wheat. If the minimum protective level be taken as the criterion, it would appear that wheat germ was almost three times as potent as whole wheat. The various samples of yellow corn were assayed because it was felt that differences in such conditions as moisture, temperature, etc., during the growing season might have some effect on the vitamin B<sub>4</sub> content. The data do not show any appreciable difference in incidence of paralysis among the groups on the different types of yellow corn, but better growth was obtained in the groups receiving the Texas corn than in those receiving Wisconsin corn. However, only very limited significance can be attached to growth rates in experiments of this kind since it is not definitely known that the basal ration is complete in all factors other than vitamin B<sub>4</sub>.

Peanuts appeared to be about twice as potent in vitamin B<sub>4</sub> as the best of the cereals, and among all the materials tested were excelled only by the grass. Some difficulty was experienced in drying the grass in such a way as not to decrease its B<sub>4</sub> content; hence the practice of adding NaCN to inhibit oxidative enzymes during the drying process was adopted. This method was also used with other materials, as indicated in table 2. The figures given here for brain tissue do not



indicate any advantage for the cyanide treatment, but it was found that only by this method could reproducible results be obtained and that brain tissue dried in the ordinary manner was very variable as to B<sub>4</sub> content. The results obtained with the sample of spinal cord seem to indicate that this tissue does not share the relatively high potency of brain. There was some variation in the vitamin B<sub>4</sub> potency of the vacuum dried liver. It was reported in an earlier paper (Keenan et al., '33) that 18% of this liver was sufficient, but in these experiments 24% was required to give protection from paralysis. The fact that the basal ration used in the earlier work probably was not as low in vitamin B<sub>4</sub> as the basal ration used in these experiments needs to be taken into account. As shown in the table, pork kidney ranked as equal to pork brain, peanuts and wheat germ. Like the other animal tissues, it is subject to the disadvantage that it requires careful drying to avoid destruction of the vitamin. Since this disadvantage does not apply to peanuts, they would appear to be a good source material to use in further work.

#### SUMMARY

1. A number of food materials have been assayed for vitamin B<sub>4</sub> potency using chicks as the experimental animals.
2. Dried grass, peanuts, wheat germ, pork brain and pork kidney were found to be good sources of vitamin B<sub>4</sub>.
3. The grains tested were found to be relatively poor sources, but white corn and hulled oats were definitely superior to wheat and yellow corn.

We are indebted to Dr. David Klein of Wilson Laboratories, to Dr. A. R. Kemmerer of the Texas Agricultural Experiment Station, to General Mills, Inc., and to the Vio Bin Corporation, Chicago, for supplying materials for these assays.

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# VITAMIN B ASSAY USING RAT CURATIVE METHOD WITH MODIFIED DIETS AND ORAL ADMIN- ISTRATION OF ADDENDA

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About a year and a half ago it was found necessary in this laboratory to test vitamin B ( $B_1$ ) fractions more quickly than the rat growth method permitted. The rat curative method as developed by Smith ('30) was considered. Through a personal communication Ammerman and Waterman (data later published in *Journal of Nutrition*, '35) suggested their modification of the Smith diet, the preparation being simple and practical. It consisted of:

	%
Casein (technical)	16
Osborn and Mendel salt mixture	4
Cod liver oil	2
Unfiltered butter	9
Cornstarch	60
Autoclaved dried bakers' yeast	9

Ammerman and Waterman had started rats weighing 100 to 125 gm. on this diet but in order to shorten the method in our laboratory rats 70 to 75 gm. in weight were used. Over 50% of the animals died without developing polyneuritis. In looking over the literature it was found that Sandels ('30) had used a basal diet containing 15% of bakers' yeast (autoclaved 3 hours at 125°C.). The rats on the basal diet alone died within 25 to 40 days rarely showing symptoms of polyneuritis while the rats on this diet plus 0.2 and 0.4 gm. of yeast or the alcoholic extracts from 0.4 to 0.8 gm. of whole wheat had all developed polyneuritis. Sebrell and Elvove

('31) had produced polyneuritis on a diet containing 15% autoclaved brewers' or bakers' yeast (autoclaved  $2\frac{1}{2}$  hours at 15 pounds pressure) and Heyroth ('32) developed polyneuritis on a diet containing 10% Harris' autoclaved brewers' yeast, but he had obtained rats from an outside commercial source. Birch and Harris ('34) had used 10% autoclaved brewers' yeast plus 0.4% brewers' yeast in the diet to produce polyneuritis.

The bakers' yeast in the Ammerman and Waterman diet was autoclaved for 6 hours at 15 pounds pressure. As Sandels' rats on 15% bakers' yeast autoclaved at 125°C. for 3 hours died within 25 to 40 days with no polyneuritis, it was assumed that the unextracted casein and unfiltered butter fat contained the only traces of vitamin B in the Ammerman and Waterman diet. At this time there were some rats in the laboratory on the Sherman and Spohn as modified by Chase ('31) diet plus a vitamin B extract supplying about one-half the vitamin B requirement for protection and gain of 3 gm. per week. Approximately all of these rats developed polyneuritis. It was decided to add to the Ammerman and Waterman diet an amount of yeast supplying about one-third of the vitamin B requirement for growth of 3 gm. weekly. To 100 pounds of diet 400 gm. of brewers' yeast<sup>1</sup> (containing 13.3 Sherman vitamin B<sub>1</sub> units per gram) were added. The food intake of rats from 30 to 50 days on this diet averaged 2.9 gm. daily (maximum amount eaten 3.8 gm., minimum 2.0 gm.). As the diet contained 0.12 Sherman vitamin B unit per gram the animals received 0.35 Sherman vitamin B unit daily (maximum 0.46 unit, minimum 0.26 unit) or approximately one-third of the vitamin B requirement for protection and gain of 3 gm. in weight per week. Rats were run on this diet, the Smith diet and the Ammerman and Waterman diet at the same time.

The incidence of polyneuritis was much greater on the modified diet as is shown in table 1. There was only 31.1% discards on the modified diet as against 54.8 on the Smith diet and 55.7 to 58.1 on the Ammerman and Waterman diet. In a

<sup>1</sup> Dried dehydrated yeast from Northwestern Yeast Company, Chicago, Illinois.

few cases, the rats had four, five or six polyneuritic attacks and as many as nine or ten on the modified diet. The next problem was to prove that the assay run on these polyneuritic rats with oral administration of the addenda gave comparable results with those obtained on the Sherman growth method.

The rats were not used for test until they reached the spastic stage of polyneuritis—a circular movement or a rolling over of body upon being rotated by the tail. In many

TABLE 1  
*Incidence of polyneuritis on different diets*

DIET	TOTAL NUMBER OF RATS USED	WEIGHT OF RATS WHEN PLACED ON DIET	PER CENT RATS DEVELOPED POLY- NEURITIS	NUMBER OF DAYS BEFORE POLY- NEURITIS DEVELOPED	WEIGHT OF RATS ON DEVELOP- ING POLY- NEURITIS	NUMBER OF POLY- NEURITIC ATTACKS PER RAT
Smith	113	gm. 50 to 60	45.2	40	57	2.2
Ammerman and Waterman	131	90 to 110	44.3	39	72	1.8
Ammerman and Waterman	184	55 to 85 (Av. 66)	41.9	36	56	2.7
Modified diet (0.12 Sherman vitamin B unit per gram)	620	55 to 85 (Av. 64)	68.9	41	63	2.7
Modified diet (yeast withdrawn as soon as one or more rats became polyneuritic) <sup>1</sup>	200	55 to 85	54.0			2.1

<sup>1</sup> We attempted putting only the polyneuritic test animals on the yeast-free diet, but where there are several hundred rats running at the same time this is well nigh an impossible task.

cases due to this severe condition it took 48 to 72 hours for the rats to be actually cured; gradually improving and gaining weight in the meantime. The condition of each rat was recorded. If the dose were not adequate and the rat remained polyneuritic, Ammerman and Waterman put the rat on test again within 24 hours—in our laboratory 48 hours were allowed to elapse between feedings and 72 to 96 hours with borderline levels where there had been a corresponding

gain in weight. Fewer individual variations occurred when the animals were not started too soon, that is, before polyneuritis was sufficiently pronounced. The 4-day curative period has been the criterion for these tests, the actual duration of cure which the English workers used having been considered more variable due to Heyroth's ('32) findings.

The material was fed by a 1 cc. tuberculin syringe with the needle removed. It was inserted into the mouth of the rat—as far back into the throat as it would reach. The amount delivered was checked by weight. If the material was soluble in water a solution was made and a dosage from 0.1 to 0.2 cc.—preferably 0.1 cc.—was given. If the material was insoluble it was made into a soft paste with fuller's earth and vegetable oil and this was fed in the same way. A paste, such as yeast and water, works very nicely with the tuberculin syringe. In the case of a paste the specific gravity must be taken and the dose in cubic centimeters corrected by the specific gravity factor.

Using the International vitamin B adsorbate as a standard in the rat curative method, the potency of the unknown in Sherman and International vitamin B units was worked out by the formulae:

$$\frac{\text{Curative dose of International standard}}{\text{Curative dose of unknown}} \times (100 \text{ International vitamin B units per gram in standard})$$

$$\frac{\text{Curative dose of International standard}}{\text{Curative dose of unknown}} \times (133 \text{ Sherman vitamin B units per gram in standard})$$

The curative dose is that dose which effects a cure in 80% or more of the animals; the curative dose of the unknown is that which produces the same percentage of cures as the standard. The factor 133 in the second formula is the potency of the International adsorbate in Sherman units which we get in this laboratory using the Sherman rat growth method—it is a conservative figure as some laboratories report as high as 200 to 300.

The rat curative method has been used in assaying many types of material such as water-soluble concentrates, insoluble adsorbates, yeasts of greater potency than 40 Sherman units

TABLE 2

*Comparison of results in vitamin B assay using rat growth and rat curative methods*

## A. Rat growth method

NUMBER OF RATS	DOSE	GAIN IN WEIGHT PER 28 DAYS
Unknown		
8	<i>mg.</i> 4.0	<i>gm.</i> -17 (polyneuritic)
7	8.0	+15
7	12.0	+29
International vitamin B adsorbate		
7	5.0	- 5
7	7.5	+14
7	10.0	+25
Negative controls		
7		Died within 25 to 40 days without devel- oping polyneuritis

Results: Slightly more than 125 Sherman vitamin B units per gram ( $\frac{1000}{8.0}$  or  $\frac{7.5}{8.0} \times 133$ ). Slightly more than 83 International vitamin B units per gram ( $\frac{10.0}{12.0} \times 100$ ).

## B. Rat curative method

DOSE	CURED	NOT CURED	PER CENT OF CURES
Unknown			
<i>mg.</i> 15	2	4 (2 died within 24 hours—discarded)	50
20	5	1	83
25	6	0	100
International vitamin B adsorbate			
15.7	3	5	38
18.4	5	2 (1 died within 24 hours—discarded)	83
21.0	5	1 (severe, died within 48 hours—discarded)	100

Results: 122 Sherman vitamin B units per gram ( $\frac{18.4}{20.0} \times 133$ ); 92 International vitamin B units per gram ( $\frac{18.4}{20.0} \times 100$ ).

per gram, and vitamin paste mixtures. Where a finished report in Sherman units was required a check assay using the rat growth method was run as is shown in table 2.

Two levels close together—15.7 mg. and 18.4 mg.—were run on the standard to make interpretation of results more accurately. If the rats were in a severe condition the percentage of cures on the 15.7 mg. level was below 80; if the

TABLE 3  
*Vitamin B assay using rat curative method with minimum number of animals on unknown*

Rat curative method			
DOSE	CURED	NOT CURED	PER CENT OF CURES
Unknown			
mg.			
1.9	0	5	0
2.2	0	3	0
2.5	0	5	0
2.8	0	3	0
3.5	1	4	20
4.0	2	1	67
5.0	2	2 (severe condition)	50
6.0	5	0	100
7.0	4	0	100
International vitamin B adsorbate			
12.2	2	4	33
15.7	3	4 (1 died within 24 hours—discarded)	50
18.4	9	4 (2 died within 24 hours—discarded)	82

The results on the 5.0 mg. level were discarded and the potency reported between 408 and 612 Sherman vitamin B units per gram ( $\frac{18.4}{6.0} \times 133$  and  $\frac{18.4}{4.0} \times 133$ ) or between 307 and 460 International vitamin B units per gram ( $\frac{18.4}{6.0} \times 100$  and  $\frac{18.4}{4.0} \times 100$ ).

rats had been started too soon the percentage was more than 80. If a minimum number of rats were used—three or four rats to a level—variable results were obtained as is shown in the assay on a research sample in table 3.

Over 325 samples have been tested in this laboratory by the modified Smith rat curative method with the oral admin-



istration of the addenda. On approximately 10% of these samples which required a report in Sherman units a check assay using the rat growth method was run. The percentage of difference between the two methods was from 2.1 to 24.4—average 9.1.

#### DISCUSSION

The number of discards in this laboratory on the Smith diet and the Ammerman and Waterman diet seems higher than reported by others. Possibly it is entirely dependent on the animals used. Ammerman and Waterman claim their animals on the breeders' diet reach the weight of 100 to 125 gm. between 40 and 55 days—in this laboratory it takes 55 or more days for our rats to reach this weight. The disadvantage of using heavier rats is the time item. If higher results are obtained on a diet containing a definite amount of vitamin B this can be obviated by running a standard at the same time which is as necessary in the Sherman rat growth method as in the Smith rat curative method. In comparing one set of results obtained on the rat growth method in this laboratory with results in another laboratory we found that our growth rate was much lower than the other laboratory, but the final results worked out by the formula using the International adsorbate as a standard were the same. In this laboratory we have gotten consistently for the past 3 years only 133.3 Sherman vitamin B units per gram on the International standard adsorbate, thus making our conversion factor from International to Sherman units 1.33 where others got it at 2 or 3; Waterman and Ammerman ('35) gave approximately 2. This shows how impossible it is for all laboratories to use one and the same conversion factor in any bio-assay.

#### CONCLUSION

The Smith rat curative method with a modified diet is practical and within the limits of accuracy for bio-assays if the following points are adhered to: 1) the standard must be run at the same time as the unknown; 2) a certain critical

judgment is necessary in taking rats definitely polyneuritic; 3) the number of animals should not be less than that used in the rat growth method, six or more rats to each level and not less than two levels; 4) where an animal is used repeatedly for test, 48 hours should elapse between feedings even if the animal becomes polyneuritic within 24 hours; on borderline levels with a corresponding gain in weight 72 to 96 hours are necessary; and 5) it is better to make the interpretation of curative dose at a level where 80% or more of the rats are cured; at borderline levels of less than 80% cures there will be greater individual variations. Until the individual laboratory has developed its technic sufficiently it is advisable to run check assays with another familiar method such as the rat growth method or pigeon maintenance method.

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# THE EFFECTS OF GLUCOSE, FRUCTOSE AND GALACTOSE ON KETOSIS, PRODUCED BY ANTERIOR PITUITARY EXTRACT AND BY PANCREATECTOMY

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TWO FIGURES

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Evidence is growing that the different hexoses do not have equal physiological values. C. Voit as early as 1891 found that glucose and fructose were better glycogen formers in the dog than galactose, and this was confirmed by Weinland in 1899. Lusk ('15) reported that the dynamic effect of glucose, sucrose and fructose increased in the order named when given in equal doses to normal dogs. Galactose and lactose lagged behind, the former possibly because of its slower conversion after absorption, the latter because it was not readily digested. More recent evidence places galactose at the head of the list of hexoses in the order of their convertibility to glucose in the phlorhizinized dog (Deuel and Chambers, '25) and fructose ahead of glucose in combustibility (Hornemann, '23). Galactose at certain stages of absorption also leads in rate of glycogen formation (Deuel, MacKay et al., '33) in the normal dog.

As regards the ketolytic action of different sugars Goldblatt ('25) concluded from experiments on fasting human subjects that glucose, fructose; sucrose and maltose were effective while galactose, mannose, lactose (and glycerol) were not.

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Producing ketosis in man in the same manner and also by means of a protein-fat diet, Deuel, Gulick and Butts ('32) found galactose possessed a greater lytic action than glucose. Ketonuria was depressed farther and maintained at a lower level for a longer period of time. Fructose also surpassed glucose in its ketolytic effect after fasting and appeared to be intermediate between the other two hexoses.

Butts ('34) by experiments on rats in which ketosis was produced by fasting and by administration of sodium acetoacetate found galactose most effective, lactose next and glucose least, in controlling ketonuria. Water intake was especially high after galactose.

Shapiro ('35) fed *l*(+)-lactic acid, pyruvic acid and glycerol, as well as glucose, in amounts demonstrated to be glycogenic, and found a definite ketolytic action in rats fed diacetic acid. On the other hand *d*(—)-lactic acid, acetaldehyde, ethyl alcohol and ethylene glycol did not show any appreciable glycogen formation and very little ketolytic action. She found that a definite relationship existed between the ability of a substance to form glycogen and its ketolytic action.

Chaikoff and Weber ('27) concluded that carbohydrate had no antiketogenic action in depancreatized dogs in complete absence of insulin. Selle ('27) held that administration of glucose to patients in diabetic coma often relieved the symptoms of ketonuria; but glucose had no effect on ketonemia of animals deprived of their pancreas.

The present study was suggested by the thought that pancreatic ketonuria alone may not be an adequate criterion of ketogenesis and that the comparative effects of the different hexoses should be studied in ketosis produced by still other means. Dogs were chosen as subjects and ketosis produced 1) by high fat diets supplemented by injection of the ketogenic hormone of the anterior pituitary, and 2) by pancreatectomy.

#### EXPERIMENTAL

Normal female dogs were fed 1 pint of cream daily for several weeks prior to the studies with the sugars. When a

measurable ketosis had been established administration of anterior pituitary extract was begun.

Female dogs were completely depancreatized under ether and morphine anesthesia. Subcutaneous injections of insulin were given twice daily until the wound was entirely healed. Insulin was then withdrawn until a satisfactory D: N ratio was established, which, by experiment, was found to reflect about maximum ketosis. This usually occurred on the fourth to fifth day.

The dogs were confined in metabolism cages throughout the entire period of the experiment. A mixture of toluene and thymol was used to preserve the urine. Water was given *ad libitum*.

Respiratory quotients were determined, blood samples were taken from the saphenous vein and catheterizations were made, in the order mentioned, at approximately 8.00 A.M., 12.00 NOON, 4.00 P.M. and 8.00 P.M. The exact times were always noted and all values calculated to the basis of 1 hour.

The first 4-hour period (8.00 A.M. to 12.00 NOON) was used as a control. Fifty grams of the sugar to be tested were given after the 12.00 noon catheterization and the effects on the ketosis and metabolism were studied in the afternoon. The sugar was dissolved in 100 cc. of water and administered by means of a stomach tube. To the normal dog on a high fat diet, 10 cc. of a crude pituitary extract, prepared by the method of Magistris ('32) and containing the diabetogenic and fat metabolism hormones, were injected subcutaneously at the same time as the sugars were given. The diet of the depancreatized dog consisted of ground beef steak, 20 gm. pancreatin, 40 gm. of lard and 10 gm. of bone ash. Feeding always occurred at 8.00 P.M.

Autopsies were performed on all the depancreatized dogs at the completion of the experiments to determine whether any residual pancreatic tissue remained.

Pfanstiehl C.P. galactose and fructose and C.P. glucose furnished by the Corn Industries Research Foundation were used.

The gaseous metabolism determinations were carried out with the open circuit type of respiration apparatus (Tissot). The dogs were trained to lie still with a face mask and breathe into the spirometer. The analyses of oxygen and carbon dioxide in the expired air were made on a Haldane gas analyzer.

After the withdrawal of the blood a filtrate was made as soon as possible by the method of Folin and Wu. The blood sugars were determined by Benedict's method ('28). Urinary nitrogen was determined by the usual macro Kjeldahl method, using alizarine as the indicator. Urinary sugars were determined by a combination of the Munson and Walker and Bertrand methods (Mathews, '25). Blood acetone bodies were determined by the Shaffer-Hubbard distillation method as adapted by Behre and Benedict ('26) employing, however, Hubbard's method of titration. Acetone plus diacetic acid were determined together. Urine acetone plus diacetic and  $\beta$ -hydroxybutyric acid and blood  $\beta$ -hydroxybutyric acid were determined by the method of Hubbard ('21). The yield of acetone from  $\beta$ -hydroxybutyric acid by the above method was approximately 75%. No correction was made for the 25% which was not determined. This error appears to be practically constant.

## RESULTS

### *High fat diet and anterior pituitary extract*

As many investigators have shown, it is very difficult to produce a marked ketosis in a normal adult dog maintained on a high fat diet. This was accomplished, however, by use of injections of anterior pituitary extract (Magistris, '32) subcutaneously. This extract contains the diabetogenic and fat metabolism hormones which Anselmino and Hoffman ('34) recently showed to be two separate entities. The diabetogenic hormone probably lowers carbohydrate oxidation by suppressing the secretion of insulin (Képinov et al., '34). Anselmino and Hoffman ('31) and others, have shown that the fat metabolism hormone increases fat metabolism and the production of ketone bodies. Thus, by use of these two hor-

mones, a condition was obtained which closely simulates diabetes. The condition resulting from the injection of these two hormones was rather transient, but of sufficient duration to satisfy the intended purpose. This is shown in table 1 by a typical example of an experiment, and in figure 1 by averages of a series of experiments.

In the control, receiving extract alone (fig. 1) the average blood sugar rose gradually from 65 to 110 mg. per cent and reached a maximum in 8 hours. By the next morning it had fallen to the basal level. The rise can indicate either that glucose oxidation is suppressed or that the diabetogenic hor-

TABLE 1  
*High fat diet and anterior pituitary extract. Control*

TIME	URINE N	D:N	R.Q.	BLOOD SUGAR	BLOOD ACETONE	URINE ACETONE	BLOOD $\beta$ -OH	URINE $\beta$ -OH
	gm./hr.			mg./%	mg./%	mg./hr.	mg./%	mg./hr.
8.00 A.M. basal det.	0.10		0.71	63	30	2	79	9
12.00 NOON basal det.	0.10		0.71	63	29	2	78	8
12.10 P.M.	10 cc. pituitary extract							
4.00 P.M. 1st experimental period	0.19		0.70	73	31	6	88	21
8.00 P.M. 2nd experimental period	0.11		0.68	115	72	3	125	19
8.10 P.M.	Fed 1 pint xx cream							
8.00 A.M. basal	0.10		0.71	68	32	5	78	22

mone stimulates the adrenals, as Heydeman, Duensing and Lucke ('33) believed. It is possible that the maximum change of blood sugar, blood acetone, blood  $\beta$ -hydroxybutyric acid and R.Q., in all of the experiments, was not observed, because of the necessarily long interval between determinations. Concurrently with the rise in blood sugar, the R.Q. dropped slightly from 0.71 to 0.68. The three experiments were closely concordant.

The control curves for blood ketone bodies (fig. 1) closely paralleled the one for blood sugar, making an average increase from 75 to 125 mg. per cent in the case of the  $\beta$ -hydroxybutyric acid and 26 mg. to 73 mg. per cent in the case of the blood

acetone. The urine ketone bodies increased in the controls from 7 to 23 mg. per hour for  $\beta$ -hydroxybutyric acid and from 2 to 7 mg. per hour for acetone; but did not parallel the blood ketone rise. The maximum rise came during the first 4-hour period.

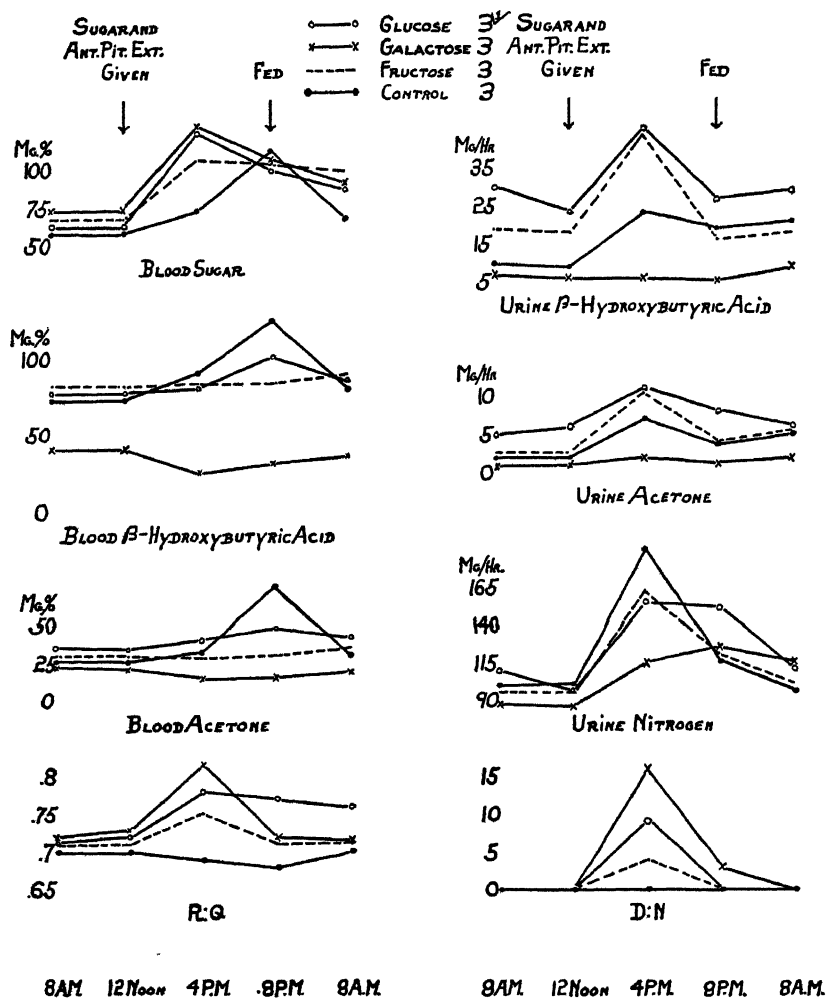


Fig. 1 Changes caused by the simultaneous oral administration of 50 gm. of sugar and subcutaneous injection of anterior pituitary extract to dogs, which were maintained on a high fat diet.

<sup>1</sup> Number of experiments included.



Since the blood ketone bodies remained at approximately the basal level during the first 4 hours, it seemed that the kidney prevented a further increase in the blood ketone bodies by excreting them as fast as they were formed. During the next period the ketone bodies were formed at a faster rate than the kidney was able to excrete them. This resulted in a marked increase in the blood, thus confirming the idea that formation and excretion may differ considerably in rate.

During the first 4-hour period after the injection of the extract, the nitrogen elimination rose from 100 mg per hour

TABLE 2  
*High fat diet and anterior pituitary extract. Glucose*

TIME	URINE SUGAR	URINE N	D:N	R.Q.	BLOOD SUGAR	BLOOD ACETONE	URINE ACETONE	BLOOD $\beta$ -OH	URINE $\beta$ -OH
	gm./hr.	gm./hr.			mg./%	mg./%	mg./hr.	mg./%	mg./hr.
8.00 A.M. basal det.		0.10		0.71	64	40	3	79	27
12.00 NOON basal det.		0.09		0.73	64	36	4	75	17
12.10 P.M.	10 cc. pituitary extract + 50 gm. glucose								
4.00 P.M. 1st experi- mental period	0.90	0.14	7:1	0.79	125	46	11	77	46
8.00 P.M. 2nd experi- mental period		0.13		0.80	116	51	4	105	21
8.10 P.M.	Fed 1 pint xx cream								
8.00 A.M. basal det.		0.10		0.77	100	40	5	70	27

to 193 mg. per hour (average) and then returned to approximately normal during the subsequent 4-hour period. Only a small part of this rise could be explained by the fact that the extract injected contained 3 mg. of nitrogen per cubic centimeter. Since the diabetogenic hormone suppresses carbohydrate oxidation, the nitrogen sparing action of carbohydrate is lowered. It is also possible that the extract directly stimulates protein catabolism. No glycosuria was observed.

Tables 2, 3 and 4, which are typical examples of individual experiments, and figure 1, which shows averages of a series of experiments, illustrates the effects that glucose, fructose and galactose have on ketosis and the general metabolism of the

dogs. Again the three experiments in each group were closely concordant.

Following the administration of glucose and extract, the blood sugar rose from 65 to 125 mg. per cent during the first

TABLE 3  
*High fat diet and anterior pituitary extract. Fructose*

TIME	URINE SUGAR	URINE N	D:N	R.Q.	BLOOD SUGAR	BLOOD ACETONE	URINE ACETONE	BLOOD $\beta$ -OH	URINE $\beta$ -OH
	gm./hr.	gm./hr.			mg./%	mg./%	mg./hr.	mg./%	mg./hr.
8.00 A.M. basal det.		0.09		0.71	69	28	3	85	18
12.00 NOON basal det.		0.09		0.71	70	28	3	85	18
12.10 P.M.	10 cc. pituitary extract + 50 gm. fructose								
4.00 P.M. 1st experi- mental period	0.44	0.18	2.5:1	0.74	101	28	10	88	49
8.00 P.M. 2nd experi- mental period		0.11		0.71	103	32	4	88	12
8.10 P.M.	Fed 1 pint xx cream								
8.00 A.M. basal det.		0.09		0.71	103	35	8	96	19

TABLE 4  
*High fat diet and anterior pituitary extract. Galactose*

TIME	URINE SUGAR	URINE N	D:N	R.Q.	BLOOD SUGAR	BLOOD ACETONE	URINE ACETONE	BLOOD $\beta$ -OH	URINE $\beta$ -OH
	gm./hr.	gm./hr.			mg./%	mg./%	mg./hr.	mg./%	mg./hr.
8.00 A.M. basal det.		0.09		0.72	68	20	1	36	8
12.00 NOON basal det.		0.09		0.73	68	19	1	35	7
12.10 P.M.	10 cc. pituitary extract + 50 gm. galactose								
4.00 P.M. 1st experi- mental period	2.79	0.12	17:1	0.83	122	15	2	15	7
8.00 P.M. 2nd experi- mental period	0.38	0.12	2:1	0.74	91	15	1.5	27	5
8.00 P.M.	Fed 1 pint xx cream								
8.00 A.M.		0.11		0.74	82	18	3	30	11

4-hour period, and then dropped to 100 mg. per cent during the next 4-hour period. The blood sugar curve following the administration of galactose and extract closely paralleled that for glucose. Fructose raised the blood sugar from 65 to only

105 mg. per cent during the first 4-hour period; but, maintained it at this level for the subsequent 4-hour period. All three of the monosaccharides sustained the blood sugar above normal for 20 hours after the administration. Many investigators have found that if a certain class of foods is withheld from the body for a long period of time, the body compensates for the deficiency by adaptation to increased utilization of another class of foods. When the deficiency food is again added to the diet, it requires a certain period of time before the body can readjust itself to utilize this food, which is now a relatively foreign substance to the body (Joslin, '28). Furthermore, in the present instance the oxidative mechanism of carbohydrate has been interfered with by the injection of the diabetogenic hormone. These facts probably accounted for the prolonged high blood sugars.

The injection of 10 cc. of pituitary extract alone raised the blood  $\beta$ -hydroxybutyric acid in 8 hours from 75 to 125 mg. per cent and the blood acetone from 26 to 73 mg. per cent. When glucose was administered along with the extract (table 2 and fig. 1), the blood  $\beta$ -hydroxybutyric acid rose, on the average, in the same time from 77 to 102 mg. per cent and the acetone from 34 to 50 mg. per cent. Fructose (table 3 and fig. 1) completely offset the effect due to the extract. The blood ketone bodies were maintained at approximately a constant level during the entire experiment. Galactose (table 4 and fig. 1), however, not only completely offset the effect due to the extract, but also lowered the blood ketone bodies below the basal level. The blood  $\beta$ -hydroxybutyric acid dropped from 40 to 25 mg. per cent and the blood acetone from 22 to 15 mg. per cent. Both rose to approximately basal level by the twentieth hour. Quite different were the effects of glucose and fructose on the ketonurias. During the first 4 hours after the administration of glucose, the urine  $\beta$ -hydroxybutyric acid rose from 23 to 45 mg. per hour and then returned to the basal level during the next 4-hour period. The high points reached were above the controls. The urine acetone rose from 7 to 11 mg. per hour during the first 4-hour period, and then re-

turned gradually to normal by the twentieth hour. The curves for urine ketone bodies (fig. 1) following the administration of fructose, paralleled closely those for glucose. Galactose held the excretion of  $\beta$ -hydroxybutyric acid to the base level and even caused it to drop slightly by the eighth hour, while the urine acetone increased only slightly during the first 4-hour period. Both returned to basal during the next 4 hours.

Galactose also exerted a greater protein sparing action than did glucose or fructose. This is shown in table 4 and figure 1. The urine nitrogen after galactose administration rose on the average from 85 to 115 mg. per hour during the first 4-hour period. It rose to 127 mg. per hour during the next period and then dropped slightly. Contrast this with the control curve. Fructose exerted a slightly greater nitrogen sparing action than glucose. The nitrogen elimination after the administration of glucose and extract rose from 95 to 155 mg. per hour during the first 4 hours and remained at this level for the next 4-hour period. It returned then to approximately normal during the subsequent 12 hours. After the administration of fructose and extract, the nitrogen elimination rose on the average to 160 mg. per hour during the first 4-hour period and then dropped to 120 mg. per hour during the succeeding 4-hour period.

A slight glycosuria was observed in all the experiments during the first 4-hour period after the administration of sugar. Galactose caused the highest glycosuria followed by glucose and then fructose.

Glucose raised the R.Q. from 0.72 to 0.78 during the first 4-hour period and maintained it above 0.76 for the subsequent 16 hours of the experiment. In the case of fructose, the R.Q. rose from 0.71 to 0.75 during the first period and then returned to basal during the next period. Galactose gave a similar reaction, but the rise from 0.73 to 0.81 was slightly higher at the end of the first 4-hour period. No simple proportionality existed between the effects on combustion and the ketolytic action, as judged by events at these 4-hour intervals.

*Depancreatized dog*

Tables 5, 6 and 7, and figure 2, show the effects of administration of glucose, fructose and galactose on depancreatized animals. The tables illustrate typical examples of individual experiments, while the figure shows the average percentage

TABLE 5  
*Depancreatized dog. Glucose*

TIME	URINE SUGAR	URINE N	D:N	R.Q.	BLOOD SUGAR	BLOOD ACETONE	URINE ACETONE	BLOOD $\beta$ -OH	URINE $\beta$ -OH
	gm./hr.	gm./hr.			mg./%	mg./%	mg./hr.	mg./%	mg./hr.
8.00 A.M. basal det.	4.62	0.87	5:1	0.76	285	26	6	50	113
12.00 NOON basal det.	0.38	0.43	0.9:1	0.72	228	24	9.5	42	31
12.10 P.M.	50 gm. glucose								
4.00 P.M. 1st experi- mental period	3.79	0.24	18:1	0.72	392	17	9	42	23
8.00 P.M. 2nd experi- mental period	6.50	0.24	27:1	0.71	285	21	16	34	38
8.10 P.M.	Fed ground beef								
8.00 A.M. basal det.	4.64	0.88	5:1	0.76	205	26	26	46	92

TABLE 6  
*Depancreatized dog. Fructose*

TIME	URINE SUGAR	URINE N	D:N	R.Q.	BLOOD SUGAR	BLOOD ACETONE	URINE ACETONE	BLOOD $\beta$ -OH	URINE $\beta$ -OH
	gm./hr.	gm./hr.			mg./%	mg./%	mg./hr.	mg./%	mg./hr.
8.00 A.M. basal det.	4.2	0.84	5:1	0.77	286	25	28	50	116
12.00 NOON basal det.	0.89	0.53	2:1	0.73	286	25	11	54	41
12.10 P.M.	50 gm. fructose								
4.00 P.M. 1st experi- mental period	2.36	0.29	8:1	0.72	412	24	12	55	38
8.00 P.M. 2nd experi- mental period	5.01	0.25	20:1	0.72	354	28	9	58	26
8.10 P.M.	Fed ground beef								
8.00 A.M. basal det.	3.56	0.63	5.5:1	0.75	281	26	22	55	96

change in a series of experiments which were fairly concordant. Computation of percentage change was used because there existed a considerable difference in the severity of the dogs' conditions following the withdrawal of insulin.

The dogs used were all good eaters, very active, friendly and in the best of health. A few of the dogs which refused food after the operation were discarded because they declined rapidly in general nutritive condition.

The curves for blood sugars (fig. 2) were practically identical for the three hexoses. They rose to practically the same level at 4 hours and then dropped to approximately normal by the eighth hour. Actually the maxima must have come earlier and may not have coincided so closely.

The excretion of sugar in the urine after the administration of glucose and fructose was highest during the second 4-hour

TABLE 7  
*Depancreatized dog. Galactose*

TIME	URINE SUGAR	URINE N	D:N	R.Q.	BLOOD SUGAR	BLOOD ACETONE	URINE ACETONE	BLOOD β-OH	URINE β-OH
	gm./hr.	gm./hr.			mg./%	mg./%	mg./hr.	mg./%	mg./hr.
8.00 A.M. basal det.	4.02	0.88	5:1	0.76	294	34	20	76	103
12.00 NOON basal det.	0.58	0.45	1.3:1	0.72	303	40	11	76	54
12.10 P.M.	50 gm. galactose								
4.00 P.M. 1st experi- mental period	7.44	0.48	16:1	0.72	440	38	15	70	50
8.00 P.M. 2nd experi- mental period	4.97	0.40	13:1	0.71	354	29	15	54	48
8.10 P.M.	Fed ground beef								
8.00 A.M. basal det.	4.28	0.85	5:1	0.76	286	25	28	50	116

period (tables 5 and 6) while the maximum excretion, following galactose administration, was observed during the first 4-hour period (table 7) thus indicating that galactose is absorbed faster (Cori, '25 a) and has a lower renal threshold (Shay et al., '31; Rowe and Chandler, '24; Folin and Berglund, '22). Only 80% of the administered sugar was recovered during the 20-hour period. The remaining 20% probably diffused into the tissues (Cori and Goltz, '25) and was gradually returned to the blood stream and excreted. If the urine had been collected for another 24 hours probably nearly all of the administered sugar would have been regained.

The D:N ratios fluctuated considerably from period to period. This variation is very characteristic of a depancreatized dog. They were usually about 5:1 in the early morning which was much higher than the Minkowski ratio of 2.8:1. This was due to the fact that 20 gm. of pancreatin, containing about 80% dextrin was given along with the ground

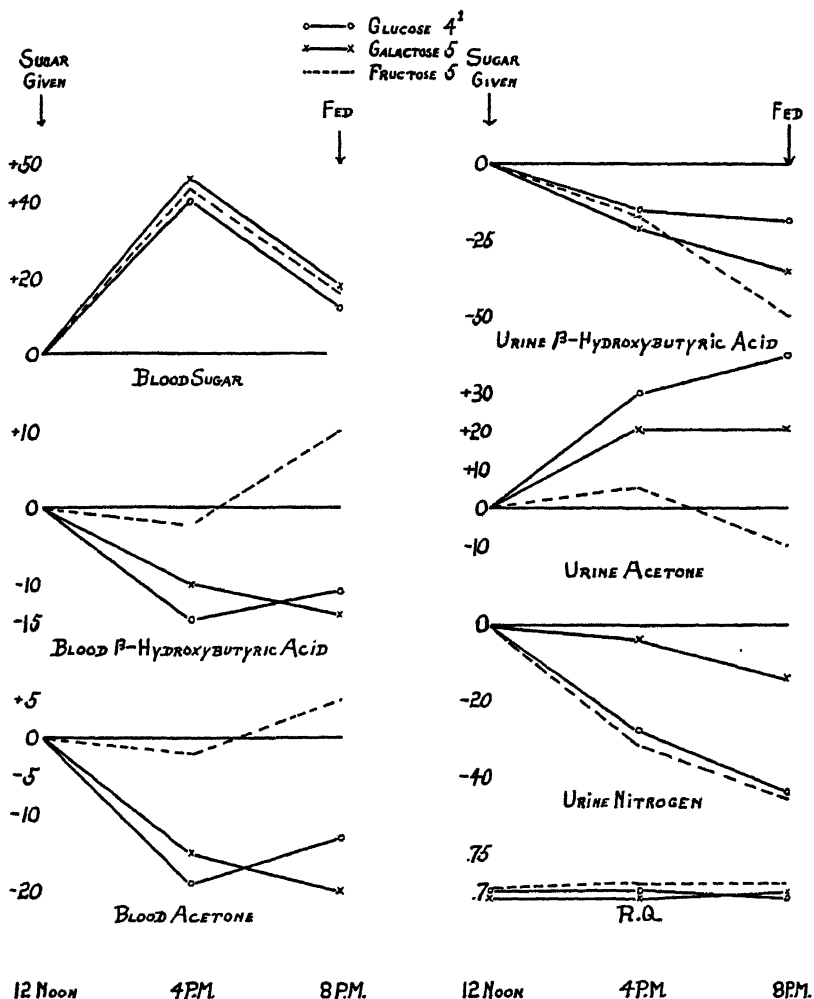


Fig. 2 Percentage change caused by the oral administration of 50 gm. of sugar to depancreatized dogs. All curves represent averages of the experiments.

<sup>1</sup> Number of experiments included.

beef steak and lard the preceding evening. When a correction was made for this extra sugar, the resulting D:N ratio was about 3.5:1, which is within the range of daily variation of a depancreatized dog.

None of the sugars was oxidized as is shown by the R.Q. which usually dropped gradually from 0.71 or 0.72 to 0.68 or 0.69 during the 8 hours following sugar administration (fig. 2). Such a variation is possibly accounted for by the ketosis and conversion of glycerol to glucose. It happens that the type experiments shown in tables 5, 6 and 7 do not show R.Q.'s quite so low as this.

Glucose caused an average drop of 15% in blood  $\beta$ -hydroxybutyric acid and 19% in blood acetone within 4 hours after administration of the sugar. Both returned gradually to basal within 20 hours (fig. 2 shows only 8 hours). The  $\beta$ -hydroxybutyric acid was still depressed 11% and the acetone 13% at the eighth hour. Galactose showed the same antiketogenic effect as in the anterior pituitary extract dog on a high fat diet, except that the action was slightly delayed.<sup>2</sup> The blood  $\beta$ -hydroxybutyric acid was lowered 14% and the blood acetone 20% (fig. 2). This level was reached at the eighth hour and maintained through the twentieth hour (table 7).

Fructose caused a slight drop in blood  $\beta$ -hydroxybutyric acid and acetone by the end of the first 4-hour period. This was followed by a rise of 10%<sup>3</sup> above basal in the case of  $\beta$ -hydroxybutyric acid and 5% in the case of acetone. Both returned to the basal level by the twentieth hour (table 6).

All three of the sugars caused a drop in urine  $\beta$ -hydroxybutyric acid which reached a maximum within 8 hours.<sup>4</sup> Glucose lowered this constituent 20%, galactose 35% and fructose 50%, on the average. Glucose increased the urine

<sup>2</sup> Three of the five experiments showed a marked drop at 4 hours and two of these a further drop at 8 hours; the other two continued at base level until 4 hours, then dropped still farther than the three, at 8 hours.

<sup>3</sup> Three of the five experiments showed no or a very slight rise at 8 hours, the other two rose to 24 and 13% above basal.

<sup>4</sup> Only three individual exceptions to this course—two with glucose and one with fructose all of which showed a return to or toward basal at 8 hours.



acetone plus diacetic 30% during the first 4-hour period and it rose to 40% during the subsequent 4-hour period. Galactose caused a 20% rise in urine acetone plus diacetic during the first 4-hour period and maintained it at this level for the next period. Fructose raised this fraction slightly during the first 4-hour period, but lowered it to 10% below the basal level during the next period. The high urine  $\beta$ -hydroxybutyric acid and acetone observed in the morning was due to the lard which was fed the previous evening. This was not observed when lard was withheld from the diet.

All three of the sugars showed a nitrogen sparing action. Glucose caused an average drop in nitrogen elimination of 44%, fructose 46% and galactose 12%. The maximum sparing action was observed, in all three cases, at the end of the second 4-hour period (fig. 2). Galactose did not show any superior sparing action in the depancreatized dogs as it did in the high fat dogs. Fructose and glucose showed a comparatively equal sparing action. The high urine nitrogen observed in the early morning specimen was due to the food which was given the preceding evening (tables 5, 6 and 7).

#### DISCUSSION

##### *High fat and anterior pituitary extract*

Since glucose, which may be considered the most physiological of the sugars, forms the chief, if not the only reducing sugar of the blood, one would think it ought to have the best antiketogenic power. However, such is not the case. The antiketogenic effect of glucose was quite moderate. Galactose exerted the greatest ketolytic action of any of the three monosaccharides and fructose intervened between the two. These results are entirely in accord with those of Deuel, Gulick and Butts ('32), who worked with humans and those of Butts ('34) who worked with rats.

Most investigators have used the change in ketonuria as their criterion for determining the ketolytic action of various substances. The writers do not think this is an adequate way

to determine such an effect because it was found that the amount of ketone bodies in the urine varied often in the same direction as in the blood, but sometimes also in the opposite direction. This was well shown in the determinations exhibited in tables 2, 3, 4 and fig. 1. No positive explanation for the peculiar rise in urine ketone bodies after the administration of glucose and fructose can be offered. It is not likely that it is wholly a washing out phenomenon due to the diuresis which is known to accompany glycosuria; otherwise it would certainly have been observed in the depancreatized dog (fig. 2) after the ingestion of sugar. Hubbard and Wright ('24) have shown that the variation in excretion of ketone bodies does not depend upon variations in urine volume. A considerable rise was observed in the control experiments; therefore, the effect may be due entirely to the extract.

Why galactose has the best antiketogenic effect is a matter of conjecture rather than demonstration in these experiments. Deuel et al ('32) claim that it is due to the fact that galactose maintains the tissue sugar at a higher level for a longer period of time than either glucose or fructose, and also that it is a better glycogen former than was previously supposed. We have no evidence that this was the case in the experiments under discussion because liver and tissue glycogen and the concentration of sugar in the tissues before and after administration, were not determined.

The sugars seem to act in proportion to their diffusibility. It is well known that the renal threshold is much lower for galactose than the other sugars, and that it is most readily absorbed from the intestine. Glucose is next in the order of absorption and fructose last (fig. 1, D:N). In view of this, galactose probably arrives at the site of ketone body production (Macallum, '30) most rapidly, followed by glucose and fructose in that order. Whether they act in order of combustibility or power of combination to decrease ketosis is not yet certain.

*Depancreatized dogs*

With regard to antiketogenic effects in the depancreatized dogs, the findings were particularly astonishing in the light of some of the other work in this field. Chaikoff and Weber ('27) found that glucose had no effect on ketone body excretion in depancreatized dogs and Selle ('27) came to the same conclusion using the blood ketone bodies as his criterion. The present experiments show that glucose exhibited a ketolytic action, in spite of the fact that the sugar was not oxidized.

According to Shaffer's theory of antiketogenesis, carbohydrate has to be oxidized before the formation of ketone bodies is interfered with. This cannot be applied to the results under discussion because there was no oxidation of carbohydrate as indicated by the R.Q. Intermediate determinations bear out this statement.

From these experiments, one can rightfully say that carbohydrate oxidation is not the only explanation of an antiketogenesis. In another communication (Murlin, Nasset, Murlin and Manly, '36) from this laboratory, it is shown that the administration of sugar lowered the ketosis markedly in human subjects on high fat diets, in some instances reducing it almost to the vanishing point even when the R.Q. did not rise appreciably. The authors are of the opinion that under these circumstances the sugar went to fill up the depleted glycogen stores and was not burned. This phenomenon is also well exemplified in the experiments where the dogs on the high fat diet were given galactose (fig. 1). At the eighth hour after the administration of galactose, the average R.Q. had returned to the basal level of 0.72, indicating that very little, if any, sugar was being oxidized at this determination. A marked ketolytic action, however, was observed between 8.00 p.m. and 8.00 a.m. On the other hand, at the eighth hour after glucose administration, the R.Q. indicated that sugar was still being oxidized, yet there was a much smaller ketolytic effect.

Probably a close relationship exists between the rate of glycogen formation from the three monosaccharides and the ketolytic action which they exert. The antiketogenic effect

following the ingestion of glucose reached a maximum about the fourth hour and then gradually returned to normal. Deuel et al. ('33) found that the liver glycogen of dogs was greater 6 hours after administration of glucose than it was in animals which retained a corresponding amount of galactose; however, the liver glycogen in animals killed at intervals of 12 to 72 hours after sugar feeding, was higher in the dogs which had been fed galactose. This hexose caused a gradual lowering of ketone bodies which reached a maximum about the eighth hour.

Experiments on glycogen formation in the dog cannot be accomplished satisfactorily in short periods.

Cori ('26) also Feyder and Pierce ('35) have shown that fructose forms liver glycogen at a faster rate than glucose. When determinations were made 2 hours after the ingestion of fructose in the present work, a marked ketolytic effect was observed, which returned to the basal level by the fourth hour; but sufficient evidence has not been accumulated to warrant a definite statement that the maximum always occurs at this time.

Many authors are of the opinion that the depancreatized dog is capable of forming some glycogen, at least temporarily. Shapiro ('35) has shown that only glycogen formers exerted ketolytic action in the rat. It appears, therefore, that the formation of glycogen may be an important factor in the control of antiketogenesis in these experiments. In the present work it was considered more important to compare effects of different sugars on the same dog than to sacrifice them for glycogen and tissue sugar.

Deuel et al. ('32) claimed that galactose maintained the tissue sugar at a higher level, for a longer period of time, than either of the other two monosaccharides. It is not likely that this could apply to these experiments, because glucose, which had very little antiketogenic effect in the high fat dog, exerted a ketolytic effect equal to or slightly greater than galactose in the depancreatized dog. Yet the blood sugar curve for the three monosaccharides closely paralleled each

other and galactose was eliminated more rapidly than the other two.

The fact that galactose and glucose are aldehydes and fructose is a ketone, may be the reason for their greater anti-ketogenic effects in the depancreatized dog even though all are converted to glucose eventually. Ringer ('14), Shaffer ('21) and others, believe that it is the aldehyde group which combines with the ketone bodies in such a way that they are rendered oxidizable. Since glucose exhibited very little anti-ketogenic effect in the dog fed on a prolonged high fat diet and given anterior pituitary extract, such an explanation would not hold for this animal. However, it is possible that the depancreatized dog is capable of oxidizing a certain percentage of ketone bodies in its system when a large amount of an aldehyde sugar is present. Joslin ('28) has suggested that the depancreatized dog is capable of producing something which brings about a certain chemical relationship between the ketone bodies and the aldehyde sugars. This enables the depancreatized dog to oxidize the ketone bodies to a much greater extent than does a diabetic man. The increased carbohydrate concentration in the tissues may also have exerted a fat sparing action which is the basis of Macallum's ('30) theory of anti-ketogenesis.

The urine  $\beta$ -hydroxybutyric acid in this set of experiments reflected very well the blood changes except in the experiments where fructose was administered. At the eighth hour after fructose was given, the urine  $\beta$ -hydroxybutyric was markedly lowered, while the blood  $\beta$ -hydroxybutyric was above the basal level. This seems to indicate that there was a retention of the acid by the kidney.

Since galactose in these experiments did not show as superior a nitrogen sparing action as it did in the high fat dogs, and yet produced a marked lowering of blood ketones, the anti-ketogenic effect and nitrogen sparing action of the sugars are not necessarily accomplished by the same mechanism.

## CONCLUSIONS

1. Galactose has been demonstrated to possess a greater ketolytic action than glucose in the dog, on ketosis due to a prolonged high fat diet and injection of anterior pituitary extract. Fructose was intermediate. Likewise, the nitrogen sparing action falls in the same order, galactose, fructose, glucose.

2. Glucose and galactose possessed approximately equal ketolytic action in the depancreatized dog—glucose being slightly better, although neither was oxidized.

3. Fructose possessed no ketolytic action in the depancreatized dog up to 8 hours following ingestion.

4. The nitrogen sparing action of fructose and glucose were greater than that of galactose in the depancreatized dog.

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# COMPARATIVE EFFECTS OF GLUCOSE, SUCROSE AND FRUCTOSE ON KETONE SUBSTANCE PRODUCTION IN PHLORHIZINIZED DOGS

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TWO FIGURES

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## INTRODUCTION

Relatively little information is to be found in the literature concerning the comparative effects of different sugars on ketonuria and ketonemia due to phlorhizin. Apparently the only work concerning this problem directly is that of Horne-mann ('23) and of Wierzuchowski ('25) who found no difference between glucose and fructose in removing the ketosis of completely phlorhizinized dogs. Wierzuchowski noted also that there was nitrogen sparing action and complete abolition of ketonemia without a compensatory after excretion of the ketone bodies.

Considerably more data are available in reference to the effects of various sugars on ketonuria and ketonemia produced by technics other than phlorhizin. These have been reviewed in the paper preceding this one.

From work on dogs in which ketosis was induced by high fat diet and injection of anterior pituitary extract, Clark and Murlin ('36) demonstrated that a ketolytic order of galactose, fructose and glucose held in this preparation. At the same time they demonstrated that the ketolytic order of the same sugars in totally depancreatized dogs became glucose, galactose and fructose.

<sup>1</sup> The authors are indebted to the Corn Industries Research Foundation for the fellowship, held by them successively, which made this work possible.

The protein-sparing action of sugar in phlorhizinized dogs was noted by Ringer ('12), Benedict ('23) and Gaebler and Murlin ('25). A protein sparing action for galactose, fructose and lactose was noted by Deuel and Chambers ('25), and Wierzuchowski ('27) demonstrated that it ran parallel with decrease in ketonuria. The need for simultaneous consideration of nitrogen sparing action in conjunction with ketolytic action was demonstrated by Shapiro ('35) in her work on sodium aceto-acetate fed rats. She found that ethyl alcohol gave no ketolytic effect when the nitrogen excretion was within the normal limits, but in certain instances when the urinary nitrogen was high, there was a decrease in ketonuria. This was assumed to be due to a stimulation of the nitrogen metabolism by the alcohol and the ketolytic action of the extra protein burned, an effect noted by Benedict and Österburg ('15) and others.

It was the purpose of the present work to investigate the relative effects of the sugars, glucose, fructose and sucrose on the ketosis of completely phlorhizinized dogs.

#### EXPERIMENTAL

Female dogs in which phlorhizin diabetes was produced by the daily subcutaneous injection of recrystallized phlorizin suspended in sterile olive oil were used as subjects. The results to be reported are based on two series of experiments, the first in which phlorizin diabetes was induced according to the method of Sansum and Woodyatt ('15) using epinephrine at 3-hour intervals beginning with the inception of phlorizinization, and the second in which the injection of epinephrine was modified to two injections on the third day of phlorizinization. In both series phlorhizin injections were given prior to feeding at approximately 8.00 P.M. and the procedure started 4 days before the beginning of an experiment. In the first series the dogs were fed a calculated amount of food proportional to the surface area formula of Cowgill and Drabkin ('22) in an attempt to equalize the degree of ketosis in dogs of different weights. This was abandoned in

the second series, all dogs being fed 250 gm. of ground meat, 5 gm. of bone ash and 15 gm. of lard.

During the progress of an experiment, the animals were kept in metabolism cages from which the urine could be collected quantitatively. Water was given *ad libitum*.

As will be pointed out later, the method of conducting an experiment differed in the two series. Individual experimental days, divided into 4-hour periods beginning at 8.00 A.M. and ending at 8.00 P.M., were the same in both series. At the end of each 4-hour interval urine samples were obtained by catheterization, the exact time being noted so that rate calculations could be made, and blood samples were obtained from the saphenous vein. On each urine sample determinations of nitrogen (Kjeldahl) and sugar (Munson and Walker) were made. In the first series beta-hydroxybutyric acid and acetone were determined by Hubbard's ('21) method. In the second series Van Slyke's ('17) method for total acetone was substituted for the Hubbard method. Blood sugar was determined by Benedict's ('28) method. Beta-hydroxybutyric acid and acetone in the blood were determined in the first series by the Shaffer-Hubbard distillations as adapted by Behre and Benedict ('26), but using the Hubbard titration instead of the colorimetric method. In the second series Van Slyke and Fitz's ('19) method was used in determining total acetone.

The sugars used in this experiment were d-glucose supplied by the Corn Industries Research Foundation, sucrose and fructose C.P. Pfanstiehl. In the first series glucose and sucrose were given in doses of 50 gm. in 400 cc. of water by stomach tube immediately following the 12.00 M. sample. In the second series fructose and glucose were given in the same way but successively in doses of 25 and 15 gm. in 150 and 100 cc. of water, respectively.

## RESULTS

Before presenting the effects of the different sugars it is necessary to explain the terms used in the tables. Changes in ketonuria can hardly give the whole picture of any ketolytic effect, for the reason that while a certain amount of ketone

bodies is being excreted the level in the blood may be changing. Nor would the change in ketonemia alone give expression to the entire ketolytic effect. Ketone level in the blood expressed in terms of milligrams per cent denotes merely a certain fullness, as one might speak of railroad cars as full, half full, quarter full, etc. To get the total amount in transit one must know the number of cars, or, as in the present instance, the blood volume.

Having obtained the blood volume<sup>1</sup> by the vital red injection method (Peters and Van Slyke, '32) total ketosis, representing the amount of ketone bodies in circulation at any given time, is obtained as the product of the blood volume and the per cent concentration of ketone bodies as determined. Standing alone these figures are not significant of the true ketone body production of the animal, since no account is taken of the amount that is excreted in the urine. Since at body temperature ketone bodies are completely soluble, it is assumed that there will be an equilibrium between the blood and the tissues with respect to these substances, and that the kidneys fairly promptly will excrete all that reaches the organs since there is apparently no threshold value. Total ketosis and ketonuria are combined by taking the change in total ketosis from period to period, representing either an increase or a decrease, reducing this change to terms of acetone formed or burned per hour by dividing by the time interval, and adding this to the milligrams of acetone excreted in the urine per hour. Calculated according to the formula

$$\frac{\Delta \text{ total ketosis in mg.}}{\text{time in hours}} + \text{urine ketones in mg./hr.}$$

the result is called ketone substance production and represents more nearly the amount of ketone substances formed in the animal in any given interval of time.<sup>2</sup> The ketolytic effect

<sup>1</sup> In more than two-thirds of the animals. In the others blood volume was taken as 11% of the body weight.

<sup>2</sup> It is realized of course that this method of calculation overlooks the ketone substances which may not yet have reached the blood stream. While inclusion of the muscular mass and liver at, say, 50% of the body weight, would give larger numbers, the uncertainty of equal distribution in all this mass as compared with blood volume would be greatly increased.

of the different sugars, then, is based on ketone substance production as will be demonstrated in the following tables.

*Variation in ketone substance production*

It has been established from the control experiments in each series that there is considerable variation in the ketosis developed. This variation seems to be dependent upon a number of conditions, any one or all of which may play a part in a given experiment. Among those conditions are included the number of times an animal has been used for experimental work. Dogs phlorhizinized but once develop a higher degree of ketosis than dogs used several times. Apparently a degree of tolerance is developed by which the animal is able to eliminate or burn ketone bodies which reach a certain level in the blood. Goldfarb and Himwich ('33) have suggested that ketone substances may be oxidized in the presence of lactic acid. This implies that the degree of tolerance is dependent upon the amount of lactic acid present in the tissues. Another factor seems to be the nutritional state of the animal. It has been shown by Junkersdorff ('25) that ketonuria is more intense in well-fed phlorhizinized dogs than in fasting dogs. As has already been stated, an attempt to standardize the feeding was abandoned since variation was not eliminated.

A fourth factor which tended to increase the variation was the fact that the ketone substance production during the control day following administration of sugar was often higher than during the control day preceding the sugar (compare second control day, urine acetone table 2). In a 4-day experiment this would mean that the control values preceding the second sugar would be considerably higher than those preceding the first sugar.

The control periods revealed likewise a diurnal variation which, however, was not perfectly consistent. The majority of dogs showed a higher ketone substance production during the third period (4 to 8 P.M.) than in any other period during the day. From this high level the ketone substance production either decreased (first control period, table 4), or remained

fairly constant during the following period (first control period, table 3). Other controls demonstrate the more ideal conditions, namely a steady increase during the whole day.

Since there is such a wide variation in the degree of ketosis developed in different dogs, the results in the second series of experiments are based on control periods immediately preceding each experiment as indicated in tables 2, 3 and 4 and not on an isolated series of controls, as indicated in table 1.

TABLE 1  
*Effect of glucose and sucrose on ketone substance production. Series I*

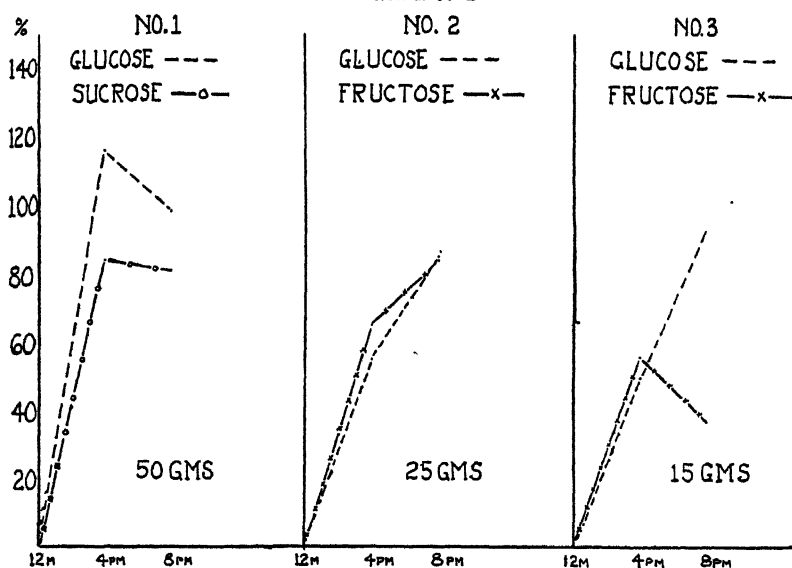
TIME	NITROGEN	URINE SUGAR	D: N	URINE ACETONE	BLOOD ACETONE	BLOOD SUGAR	KETONE SUBSTANCE PRODUCTION
Average of five controls							
8.00 A.M.	<i>gm./hr.</i>	<i>gm./hr.</i>		<i>mg./hr.</i>	<i>mg.%</i>	<i>mg.%</i>	<i>mg./hr.</i>
12.00 M.	0.418	1.26	3.00	43.7	26.9	41.3	58.2
4.00 P.M.	0.382	1.08	3.09	77.3	36.7	35.8	112.0
8.00 P.M.	0.391	1.04	2.92	95.8	41.7	36.0	113.3
Average effect of 50 gm. glucose given at 12.05 P.M.							
12.00 M.	0.384	1.32	3.44	17.5	22.0	37.5	6.8
4.00 P.M.	0.320	4.20	13.16	11.9	14.2	111.0	— 16.1
8.00 P.M.	0.300	2.18	9.50	4.8	16.0	51.5	1.7
Average effect of 50 gm. sucrose given at 12.05 P.M.							
12.00 M.	0.467	1.46	2.98	22.6	27.2	33.0	37.8
4.00 P.M.	0.328	2.65	8.13	10.2	17.1	94.1	— 16.0
8.00 P.M.	0.315	1.99	6.23	10.8	15.5	60.6	18.0

*Comparative effect of 50 gm. of glucose and 50 gm. of sucrose*

The results of these experiments shown in table 1, make up the first series of experiments to be reported. The average control reported in the table was obtained from a series of five control periods run on three different dogs, only two of which were followed by sugar. The glucose and sucrose experiments represent average figures obtained from three experiments with each sugar and the comparative effect of these two sugars shown in the graph (graph 1, no. 1) is based on the average control periods. The comparative ketolytic effect

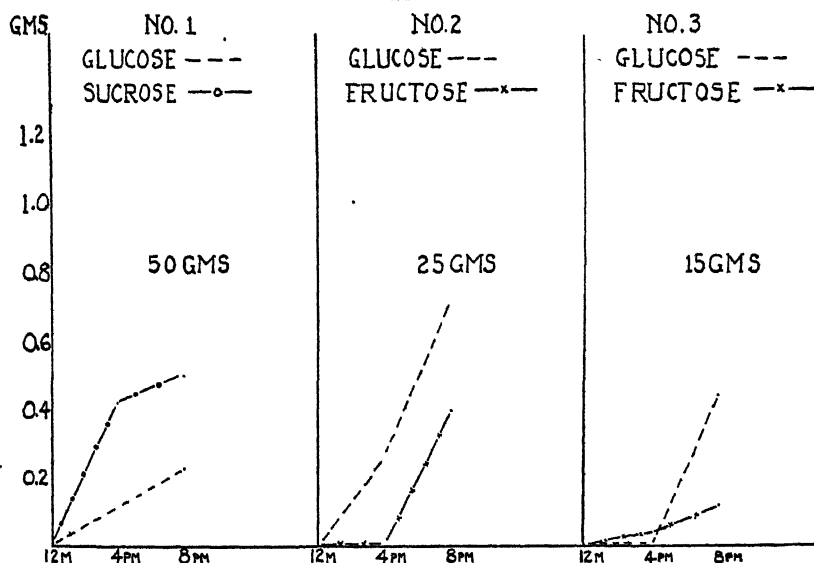
## %REDUCTION OF KETONE SUBSTANCES

GRAPH 1



## TOTAL NITROGEN SPARED

GRAPH 2



shown in graph 1, no. 1, and the comparative nitrogen sparing action shown in graph 2, no. 1, were calculated according to the method to be described in conjunction with the 25 gm. experiments. These two graphs show that glucose is superior to sucrose in ketolytic action, while sucrose is superior to glucose

TABLE 2  
*Type experiment in series II*

DATE 1936	TIME	NITROGEN	URINE SUGAR	D: N	URINE ACETONE	BLOOD ACETONE	BLOOD SUGAR
		<i>gm./hr.</i>	<i>gm./hr.</i>		<i>mg./hr.</i>	<i>mg. %</i>	<i>mg. %</i>
4/13 Control	8.30 A.M.	....	....	....	....	3.2	56.0
	12.25 P.M.	0.81	2.21	2.68	71.0	10.3	24.0
	4.25 P.M.	0.66	2.04	2.72	81.0	9.9	31.0
	8.25 P.M.	0.43	1.69	3.30	59.0	13.8	15.0
4/14 Fructose	8.40 A.M.	0.856	2.54	2.98	30.6	13.8	37.0
	12.30 P.M.	0.664	1.43	2.16	77.3	17.7	30.0
15 gm. fructose in 100 cc. H <sub>2</sub> O at 12.35 P.M.							
4/15 Control	4.30 P.M.	0.523	2.84	5.44	34.7	3.5	80.8
	8.30 P.M.	0.388	2.09	5.39	8.7	9.6	50.5
	8.45 A.M.	0.851	2.42	2.84	34.4	18.7	43.1
	12.45 P.M.	0.616	1.57	2.54	163.0	25.1	28.4
	4.45 P.M.	0.496	1.51	3.04	171.1	25.8	20.8
	8.45 P.M.	0.481	1.43	2.96	199.1	26.7	23.0
4/16 Glucose	9.00 A.M.	0.854	2.46	2.89	85.6	22.5	44.0
	1.00 P.M.	0.685	1.59	2.31	219.0	32.2	23.6
15 gm. glucose in 100 cc. H <sub>2</sub> O at 1.05 P.M.							
	5.00 P.M.	0.600	3.14	5.24	126.0	20.9	98.1
	9.00 P.M.	0.434	1.98	4.57	29.9	14.5	46.7

in nitrogen sparing action. It is to be noted that the ketolytic action of glucose decreases during the second 4-hour period while the nitrogen sparing action continues to increase. This is contrasted with the more nearly parallel action of sucrose with respect to ketolytic action and nitrogen sparing action in succeeding periods.



*Comparative effects of 25 gm. of glucose and 25 gm. of fructose*

A typical experiment, two days of which were controls, employed with 25 and 15 gm. doses of sugar is shown in table 2.

The average results for all of the 25 gm. experiments are given in table 3 and a comparison of ketolytic action of the two

TABLE 3  
*Effect of 25 gm. glucose and fructose*

TIME	NITROGEN	URINE SUGAR	D: N	URINE ACETONE	BLOOD ACETONE	BLOOD SUGAR	KETONE SUBSTANCE PRODUCTION
Average of three experiments with glucose							
	<i>gm./hr.</i>	<i>gm./hr.</i>		<i>mg./hr.</i>	<i>mg. %</i>	<i>mg. %</i>	<i>mg./hr.</i>
8.00 A.M.	....	....	....	....	30.9	36.3	....
12.00 M.	0.569	1.91	3.33	116.4	42.4	31.9	165.3
4.00 P.M.	0.605	1.75	2.90	187.1	46.4	29.7	212.4
8.00 P.M.	0.509	1.54	3.01	180.4	45.3	28.6	182.6
8.00 A.M.	0.81	2.61	3.39	74.9	45.0	55.9	171.3
12.00 M.	0.59	1.77	3.21	131.7	57.5	27.8	204.8
25 gm. glucose in 150 cc. H <sub>2</sub> O at 12.05 P.M.							
4.00 P.M.	0.56	4.95	8.95	70.4	47.4	57.7	25.6
8.00 P.M.	0.351	1.70	5.35	12.0	44.3	38.2	8.1
Average three experiments with fructose							
8.00 A.M.	....	....	....	....	45.4	45.5	....
12.00 M.	0.677	2.15	3.33	44.8	49.4	33.2	51.8
4.00 P.M.	0.464	1.57	3.46	98.1	58.2	27.7	134.7
8.00 P.M.	0.455	1.67	3.97	145.2	69.0	28.0	224.5
8.00 A.M.	0.733	2.64	3.70	51.1	47.8	50.7	9.7
12.00 M.	0.617	1.40	2.95	79.0	49.5	33.5	84.6
25 gm. fructose in 150 cc. H <sub>2</sub> O at 12.05 P.M.							
4.00 P.M.	0.435	3.62	9.64	32.9	38.3	61.6	— 25.7
8.00 P.M.	0.296	2.32	7.29	9.9	53.3	47.1	76.9

sugars is given in table 4. Ketone substance production previous to administration of glucose is considerably higher than the ketone substance production in the corresponding period of the fructose experiment. After administration of the sugar it is noted that the ketone substance production falls in both periods of the glucose experiment and in but one period of the fructose experiment. Judging the effect of the two sugars

on the basis of reduction in ketone substances from the base level would indicate a decided superiority of glucose over fructose. It is noted, in connection with the controls, however,

TABLE 4

*Comparative reduction in ketone substance production by 25 gm. glucose and fructose*

CONTROL			EXPERIMENT					
Period	Ketone substance production	Change	Ketone substance production	Assumed level	Reduction	Total production	Total reduction	Per cent reduction
Experiment 1. Glucose <sup>1</sup>								
8-12	mg./hr. 20.3	mg./hr.	mg./hr. 136.7	mg./hr. 136.7	mg./hr.	mg. 544	mg.	
12-4	78.7	58.4	— 83.7	195.1	278.8	780	1101	84.0
4-8	28.0	— 50.7	10.4	144.4	133.9	575	535	93.0
Experiment 1. Fructose								
8-12	77.7		174.0	174.0		696		
12-4	96.7	19.0	— 105.0	193.0	289.0	773	1157	81.0
4-8	83.3	— 13.4	12.7	179.6	166.9	719	667	93.0
Experiment 2. Glucose								
8-12	362.5		227.5	227.5		910		
12-4	382.5	20.0	— 1.0	247.5	248.5	989	995	52.0
4-8	359.7	— 22.8	77.9	224.7	146.8	896	587	65.5
Experiment 2. Fructose								
8-12	362.5		164.0	164.0		656		
12-4	382.5	20.0	103.5	184.0	80.5	736	322	23.0
4-8	359.7	— 22.8	27.5	161.2	133.7	644	535	82.5
Experiment 3. Fructose								
8-12	— 25.5		24.7	24.7		99		
12-4	191.6	217.1	— 9.3	241.8	251.1	968	1001	94.5
4-8	581.8	390.2	153.1	632.0	478.9	2530	1915	76.0
Experiment 3. Glucose								
8-12	166.1		306.4	306.4		1225		
12-4	191.6	25.5	127.4	331.9	204.5	1325	819	32.0
4-8	227.2	35.6	11.4	367.5	356.1	1469	1425	97.0

<sup>1</sup> Sugar always given immediately after 12 noon.

that ketone substance production does not remain at a fixed point, the tendency being a continuous rise throughout the day. It is assumed, then, that ketone substance production

would have risen proportionately the same on each experimental day as it did during the control day. Table 4 was constructed on this assumption, adding to the base level before administration of sugar, the same differences in ketone substance production in successive periods as appeared in the control periods of the previous day. The reduction in ketone substance production, then, is measured from the assumed level, taking the difference between the assumed level and the level as found at each period following administration of sugar. Per cent reduction is calculated from the total amount formed and the total amount reduced, the total amount formed in the period previous to administration of sugar being taken into account in the per cent reduction of the 12 to 4 o'clock period. The average results of the per cent reduction in ketone substance production after administration of 25 gm. of the two sugars are represented in the graph (no. 2, graph 1). From this graph it is seen that the per cent reduction after administration of fructose is slightly greater in the first 4-hour period than after glucose. At the end of 8 hours the average reduction is approximately the same for both sugars. The reduction in ketone substance production affected by fructose falls off more rapidly than the reduction produced by glucose. This, however, is very slight and the significance is questionable.

The nitrogen sparing action, calculated in the same way as the figures in table 4, is graphically shown in graph 2, no. 2. Here it is shown that, although there is a greater ketolytic effect 4 hours after fructose, there is less nitrogen spared in the corresponding period than there is after administration of glucose. The increase in nitrogen sparing action in the second, 4-hour period in each case, coincides with a decrease in the reduction of ketone substance production in the corresponding period.

#### *Comparison of 15 gm. of glucose and 15 gm. of fructose*

All of the 15 gm. experiments are averaged in table 5 and the comparative effect of the two sugars on ketone substance

production is shown in detail in table 6. The controls in this series show results which are comparable with those of the previous series and, as before, the base level before the administration of glucose is considerably higher than the base level before administration of fructose. The decrease in ketone substance production following glucose is in the same

TABLE 5  
*Comparative effects of 15 gm. glucose and fructose*

TIME	NITROGEN	URINE SUGAR	D: N	URINE ACETONE	BLOOD ACETONE	BLOOD SUGAR	KETONE SUBSTANCE PRODUCTION
Average of three experiments with glucose							
	<i>gm./hr.</i>	<i>gm./hr.</i>		<i>mg./hr.</i>	<i>mg. %</i>	<i>mg. %</i>	<i>mg./hr.</i>
8.00 A.M.	....	....	....	....	27.9	45.8	....
12.00 M.	0.690	1.83	2.64	92.9	27.2	46.8	88.4
4.00 P.M.	0.560	1.71	3.06	125.0	39.7	50.9	178.6
8.00 A.M.	0.510	1.61	3.14	169.0	41.1	24.3	176.0
8.00 A.M.	0.800	2.38	2.97	101.7	68.5	61.2	114.0
12.00 M.	0.630	1.98	3.10	153.0	43.9	29.9	155.9
15 gm. glucose in 100 cc. H <sub>2</sub> O at 12.05 P.M.							
4.00 P.M.	0.51	2.98	5.85	100.5	40.5	56.4	88.0
8.00 P.M.	0.34	1.73	4.60	37.0	33.3	43.4	3.9
Average three experiments with fructose							
12.00 M.	0.77	2.09	2.83	125.0	40.9	37.0	130.4
4.00 P.M.	0.62	1.83	3.37	160.0	48.0	43.1	193.9
8.00 P.M.	0.54	1.81	3.41	180.3	56.9	25.1	221.9
8.00 A.M.	0.857	2.43	2.86	147.7	65.7	52.4	191.7
12.00 M.	0.68	1.73	2.51	138.8	41.0	50.3	14.7
15 gm. fructose in 100 cc. H <sub>2</sub> O at 12.05 P.M.							
4.00 P.M.	0.52	3.85	7.30	58.8	23.8	74.9	— 15.0
8.00 P.M.	0.42	1.78	4.15	44.1	30.9	58.5	75.1

order as that noted after 25 gm. of the sugar. In both this series and the previous one, it is noticed that 4 hours after fructose there is a negative ketone substance production, possibly due to the fact that the ketone substance production before administration was lower in each case than in the glucose group.

Table 6, constructed in the same way as table 4, shows the comparative effect of the two sugars on ketone substance pro-

duction for all of the 15 gm. experiments. Graphically the per cent reduction in ketone substance production is shown in graph 1, no. 3, and indicates the slight superiority of fructose

TABLE 6

*Comparative reduction in ketone substance production by 15 gm. glucose and fructose*

CONTROL			EXPERIMENT					
Period	Ketone substance production	Change	Ketone substance production	Assumed level	Reduction	Total production	Total reduction	Per cent reduction
Experiment 1. Fructose <sup>1</sup>								
8-12	86.6		83.9	83.9		335.6		
12-4	87.7	1.1	— 16.0	85.0	101.0	340.0	404	60
4-8	127.7	40.0	18.8	125.0	106.2	500.0	424.8	85
Experiment 1. Glucose								
8-12	52.1		35.9	35.9		143.6		
12-4	89.1	37.0	— 14.8	72.9	87.7	291.6	350.8	81
4-8	120.3	31.2	8.1	103.2	95.1	412.8	380.4	92
Experiment 2. Fructose								
8-12	100.6		93.8	93.8		374.2		
12-4	79.5	— 21.1	— 24.6	71.7	96.3	286.8	385.2	58
4-8	75.2	— 4.3	34.1	67.4	33.3	269.6	134.2	49
Experiment 2. Glucose								
8-12	190		269.3	269.3		1177.2		
12-4	173.8	— 16.2	79.0	253.1	174.1	1012.4	706.4	33
4-8	202.8	29.0	3.2	282.1	278.9	1128.4	1115.6	98.5
Experiment 3. Glucose								
8-12	23.3		173.0	173.0		692.0		
12-4	273.0	249.7	202.0	422.0	220.0	1688.0	880	37
4-8	205.0	— 68.0	24.9	354.0	329.1	1416.0	1316.4	93
Experiment 3. Fructose								
8-12	204		— 101	— 101.0		— 404.0		
12-4	414.5	210.5	— 7.8	99.5	107.0	398.0	430	54
4-8	463.0	48.5	71.8	138.0	+ 33.5	552.0	+ 134	+ 24

<sup>1</sup> Sugar always given immediately after 12 noon.

over glucose in the first 4-hour period. In the second 4-hour period glucose continues to reduce ketone substance production while fructose drops off markedly.

Nitrogen sparing action, graph 2, no. 3, calculated as before, indicates the superiority of glucose in the second 4-hour period. In contrast to the adjoining graph, neither glucose nor fructose produces any nitrogen sparing action in the first 4-hour period. Evidently the nitrogen sparing property is not positively correlated with ketolysis at this level of feeding the sugars. As the nitrogen sparing action of fructose appears in the second period, the ketolytic effect diminishes, in contrast to the simultaneous increase in nitrogen sparing and ketolytic action of glucose.

#### DISCUSSION

The conception of ketone substance production is offered as a better measure of ketosis than can be either ketonemia or ketonuria. It assumes obviously that the ketone level of the blood reflects very quickly changes of ketone level in the ketogenic tissues (probably the liver in the main) and that the kidney pretty promptly responds to these changes in level, since no known thresholds for the ketone substances exist. The blood content then represents some rather definite fraction of the ketones present in the whole body, and if only this fraction were known, the term ketone substance production could be corrected factorially.

Disappearance of ketone substance, almost certainly by oxidation, may be measured by the same calculation. Referring to table 4, experiment 1, it is observed that on the first day 780 mg. of ketone substances would have been formed if sugar had not been given. Administration of glucose, however, prevented the formation of that amount and at the same time caused the disappearance of approximately 220 mg. ( $136.7 + 83.7$ ) of the already-formed 544 mg. This sort of effect is indicated by negative signs in tables 1, 3 and 5. Negative ketone substance production then indicates greater disappearance (oxidation) of blood ketones than the amount excreted through the kidney in the corresponding period.

There are some instances in the tables, however, where demonstrated disappearance of ketones does not show up as a negative production. For example, in table 3, after administration of glucose the average figure does not work out a

negative production, although in table 4 the figures for the first two experiments with glucose clearly indicate that ketones have disappeared. The average ketone substance production before the administration of sugar was sufficiently high to prevent the average reduction from showing as a negative production. Judging by the low positive production at the end of 8 hours, it is probable that a negative production would have appeared in another 4 hours.

As was pointed out by Wierzuchowski ('27) the greatest ketolytic effect follows the largest dose of sugar. Although the ketolytic effect of glucose after 25 and 15 gm. is almost the same, the maximum effect occurs after administration of 50 gm. Also 25 gm. of fructose produce a greater effect than 15 gm. The fact that the ketolytic action following 50 gm. of glucose falls off during the second 4-hour period may be due to complete abolition of the ketosis in the first period since, as seen in table 1, the ketone substance production is very low before the sugar was given.

In the 15 and 25 gm. experiments the nitrogen sparing action of glucose is superior to fructose, and is roughly proportional to the amount of sugar given. The results with 50 gm., however, are contradictory both as to the order of nitrogen sparing and the amount compared with the dose of sugar given. A tentative nitrogen sparing order would be glucose, sucrose and fructose.

That the nitrogen sparing effect of sugars did not turn out to be parallel to or to bear any other definite relationship to ketolytic effect, may be ascribed to inevitable errors of determination or to an inadequate number of experiments, or both. However, this relationship has been rather assumed from the Shaffer-Woodyatt theory of keto-antiketogenesis, than demonstrated by previous investigators. Wierzuchowski ('27) came nearest to a demonstration of such a relationship when he undertook (p. 442) a 'dynamogenic balance' after glucose ingestion in the phlorhizinized dog. He found that the physiological heat value of the protein spared in a series of nine experiments came to a total of 407 Cal. Subtracting from

this the calories represented by 58% of the protein which would have been wasted as sugar, if the protein had been katabolized, there remained 171 Cal. Then calculating the ketolytic effect of the glucose as beta-hydroxybutyric acid and 'supposedly oxidized,' he arrived at the figure 174.4 Cal. He very properly discounted this coincidence for two reasons: 1) the protein moiety remaining after sugar formation almost certainly would have formed some ketone substances, and 2) there was a considerable amount of sugar unrecovered (132.5 Cal.) which must have contributed to the ketolysis, probably, "by direct chemical influence in the sense of Shaffer." A third reason obviously is the improbability that all the ketones were first converted to beta-hydroxybutyric acid before oxidation.

A similar coincidence can be found in the present experiments. Thus the following values may be calculated.

	<i>Protein spared</i>		<i>Sugar-unrecovered</i>		<i>Ketolysis as <math>\beta</math>-hydroxybutyric acid</i>	
	gm.	Cal.	gm.	Cal.	gm.	Cal.
6 Exp. Fructose	27.0	118.7	49.1	183.1	12.9	60.7
6 Exp. Glucose	31.4	138.2	47.5	178.0	18.3	85.7

Deducting 58% of the protein lost as sugar there remains 60.4 Cal. spared for fructose and 70.8 Cal. for glucose. One must not be misled by such a striking coincidence as this in the case of fructose, for it is the result of ignoring the several considerations mentioned above. What impresses the present writers in the results tabulated above is the lack of correspondence between the ketolytic effect, expressed either as grams or calories, and the sugar unrecovered in the urine. In a later paper from this laboratory an attempt is made to correlate combustion of carbohydrate with the reduction of ketone substance production brought about by administration of sugar. Meantime it may be added that nitrogen can be spared in phlorhizinized dogs by carbohydrate even though it is not burned, as demonstrated by Gaebler and Murlin ('25). The unburned fraction should also be studied in relation to ketone substance production.



## CONCLUSIONS

1. The ketolytic effect of 50 gm. of glucose is greater than 50 gm. of sucrose in phlorhizinized dogs.

2. With small doses of sugar (15 gm.) the ketolytic effect of glucose is greater than fructose. With larger doses (25 gm.) the ketolytic effect of the two sugars is practically the same.

3. A comparison of the nitrogen sparing action and the ketolytic effect of the sugars does not demonstrate any close parallelism between these two functions in the phlorhizinized dog. A tentative nitrogen sparing order is given as glucose, sucrose and fructose.

4. The greatest ketolytic effect follows the largest dose of sugar.

5. The ketolytic effect of sugar is measured by its effect on ketone substance production, a combination of ketonuria and ketonemia, the formula for which is given.

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# THE EFFECT OF MONO-, DI- AND TRICALCIUM PHOSPHATES ON REPRODUCTIVE SUCCESS IN RATS

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## ONE FIGURE

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We have previously reported the effect of different levels and ratios of calcium and phosphorus on reproductive success in rats (Cox and Imboden, '36). Reference to this earlier report (fig. 1, p. 155) indicates that relatively poor performance can be expected at high levels of mineral intake even when the Ca/P ratio is near to that ordinarily considered optimal (1.0 to 2.0). Because there has been some discussion of the effect of different types of calcium phosphate when fed during reproduction (Maynard, '33; Rottensten and Maynard, '34) we felt that information of some value might be obtained by employing the three calcium phosphates ( $\text{Ca}_3(\text{PO}_4)_2$ ,  $\text{CaHPO}_4$  and  $\text{Ca}(\text{H}_2\text{PO}_4)_2$ ) in experiments similar to those previously reported, but at high levels of intake.

## EXPERIMENTAL

The basal diet, the technic employed and the method of treating results are described in our previous report (Cox and Imboden, '36). The level of calcium employed was 2.45% of the diet; and, with the exception of the small amount (0.017%) in the basal diet, was furnished by adding each of the three calcium phosphates. At this high level of intake we felt that any differences in the three salts (which might not appear at lower levels) would be manifest.

The experimental mothers were followed for eleven reproductive cycles: the first cycle while receiving stock food, and the remaining ten on the experimental rations. The results are presented in table 1, together with comparable groups from the previous study.

#### DISCUSSION

We have advanced the thesis (Cox and Imboden, '36) that the optimal ratio of calcium and phosphorus for reproductive success is determined by the calcium level. We found, for example, that at a level of 1.225% calcium, the optimum ratio (on the basis of weight of young at 21 days) was 1.66, and at the 2.45% level, 2.0. No diets with ratios between 1.0 and 2.0, at the highest calcium level, were employed. In the present work results with diets having Ca/P ratios between 1.0 and 2.0, show that the optimum ratio at high levels of calcium intake (2.45%) is about 1.63. This level of calcium, on the basis of the average weight of young at 21 days (46.4 gm.), compared very favorably with any other calcium level, and was superior to the same calcium level (2.45%) at slightly different Ca/P ratio; e.g., Ca/P ratio, 2.0; average weight, 37.3 gm.; Ca/P ratio, 1.15; average weight, 40.0.

At high calcium levels the Ca/P ratios which give young of the largest average weight are almost identical: at 0.735% calcium level, 1.5; at the 1.225% level, 1.66; and at the 2.45% level, 1.63. The interesting fact is that low calcium levels permit a wide range of possible Ca/P dietary ratios with but little deleterious effect on reproductive performance; e.g., four diets at a calcium level of 0.49% and Ca/P ratios between 0.4 and 2.0, gave average weights of young at 21 days of from 41.3 to 46.7 gm. (Cox and Imboden, '36, table 2); whereas at high calcium intakes the possible range of ratio is much narrower (between about 1.7 to 1.2). This fact is illustrated graphically in figure 1. Similar conclusions are also apparent from a consideration of the 'success' ratings.

Because of the wide range of permissible Ca/P ratios when low levels of calcium are used, it is not to be expected that, at

TABLE 1  
*The effect of a high calcium level on reproduction in rats*

Diet no.	DIET				GESTATION				LACTATION			FACTORS EVALUATED			FINAL SUCCESS GRADE
	Salts	Per cent Ca	Per cent P	Ca/P ratio	Number completed	Number incomplete	Total living young	Number available to raise	Number completed	Number young raised	Per cent available young raised	Average weight young at 21 days	Ash content grade	Changes in weight mother	
21	..... <sup>1</sup>	2.45	0.245	10.00	2	0	19	12	0	0	0.0	....	.....	-126	0.5
22	..... <sup>1</sup>	2.45	0.490	5.00	2	0	20	12	0	0	0.0	....	.....	-40	0.8
23	..... <sup>1</sup>	2.45	0.735	3.33	18	1	124	95	12	57	60.0	19.16	85.7	-42	20.7
24	..... <sup>1</sup>	2.45	1.225	2.00	21	3	185	119	21	110	92.4	37.33	99.2	+31	40.2
33	Ca <sub>3</sub> (PO <sub>4</sub> ) <sub>2</sub>	2.45	1.50	1.63	40	0	324	216	37	165	76.4	46.37	95.3	+64	79.5
32	CaHPO <sub>4</sub>	2.45	2.06	1.15	46	1	378	251	27	117	46.6	40.01	92.50	+71	73.6
25	..... <sup>1</sup>	2.45	2.45	1.00	35	1	295	193	19	82	42.5	33.51	105.10	+22	52.8
34	Ca(H <sub>2</sub> PO <sub>4</sub> ) <sub>2</sub>	2.45	4.01	0.61	5	0	40	30	3	14	46.7	17.20	136.9	-21	6.0

<sup>1</sup> Consult Cox and Imboden ('36) for composition of salts employed.

<sup>2</sup> Inadvertently. The small difference in level is not considered significant.

such levels, great differences in the two salts,  $\text{Ca}_3(\text{PO}_4)_2$  and  $\text{CaHPO}_4$  would be observed. Although, on the basis of our earlier work, the latter salt with the lower ratio (1.29) would presumably be preferred to the former (ratio 1.94), at high calcium levels the reverse is true. The 'success' ratings were 79.5 and 73.6, and the average weights of young at 21 days

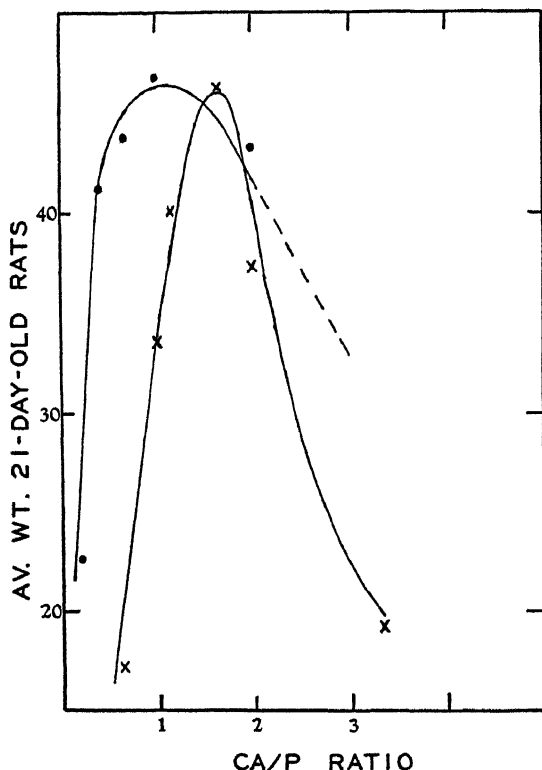


Fig.1 Average weight of 21-day-old nursing rats from mothers fed diets containing different Ca/P ratios. Diets containing 2.45% calcium are indicated by X; those containing 0.49% calcium are indicated by solid dots.

were 46.4 and 40.0 gm. for  $\text{Ca}_3(\text{PO}_4)_2$  and  $\text{CaHPO}_4$ , respectively. From comparisons on a surface area basis it was suggested (Cox and Imboden, '36) that a calcium level of 0.245% was equivalent to a human calcium intake of 1.37 gm. daily during gestation; the present level (2.45%) would therefore be equal to a daily human intake of 13.7 gm. calcium, as

the food intake of the two groups of rats was strictly comparable.

Maynard ('33) in his review on the value of calcium supplements in animal feeding could find no preponderance of evidence in favor of any one form of calcium. In his further work with Rottensten and Maynard ('34) on the effect of different calcium phosphates on bone ash and growth in rats, he could reach no definite conclusion regarding the superiority of the secondary or tertiary phosphate. The femurs of our mother rats showed 61.92, 62.22 and 61.79% bone ash for the primary, secondary and tertiary phosphates, respectively.

From table 1 it is evident that increase in dietary phosphorus resulted in progressively higher ash grades, even when the Ca/P ratio became less than 1.0. This has been discussed in our previous report, and it was pointed out that excessively high body ash content when due to an excess of phosphorus is not indicative of correspondingly high bone ash.

We consider it of additional interest to note that even at the high calcium level employed and the absence of vitamin D, the mother rats on the secondary and tertiary phosphates showed no gross signs of excessive mineral intake—either as noted during life, or at autopsy.

#### SUMMARY

Experimental rats were observed for eleven reproductive cycles to determine the effect of primary, secondary and tertiary calcium phosphates fed at a high calcium level. At a level of 2.45%, the range of Ca/P ratio which allows optimum reproductive performance is much narrower than when low calcium levels are employed, and is between 1.7 and 1.2.

At this calcium level (2.45%) the primary calcium phosphate is definitely unsuitable for reproductive success. The tertiary phosphate is slightly superior to the secondary, but this difference would probably not be apparent at lower calcium levels.

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# THE RESPONSE OF RATS, CHICKS AND TURKEY POULTS TO CRYSTALLINE VITAMIN G (FLAVIN)

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SIX FIGURES

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Goldberger and Lillie ('26) demonstrated the presence of two factors in the vitamin B complex by feeding to rats an 85% alcoholic extract of white corn as a source of vitamin B. Growth was arrested, and the animals developed a dermatitis which the authors termed 'pellagra-like.' The syndrome was cured by autoclaved yeast, or by a fuller's earth adsorbate prepared from an aqueous extract of autoclaved yeast. The curative factor subsequently became known as vitamin G or B<sub>2</sub>. Kuhn, György and Wagner-Jauregg ('33) finally crystallized vitamin G (flavin) with the aid of the adsorption technic. György ('34) soon afterward showed clearly that purified vitamin B and vitamin G (flavin) are incapable of fully satisfying the rats' requirements for the vitamin B complex. He maintained that a third factor, vitamin B<sub>6</sub>, was also necessary. Later work on the vitamin B complex has shown that the question is an involved one; Elvehjem and Koehn ('35), Birch et al. ('35), Koehn and Elvehjem ('36), Dann ('36), Elvehjem and Koehn ('36).

It was thought advisable to reinvestigate vitamin G (flavin) with the precautions now available to guard against a deficiency of other factors. By the addition of crystalline vitamin G (Lepkovsky et al., '35) it was possible to restore to normality rats rendered deficient by its absence. This served as a suitable criterion for the adequacy of the basal diet with respect to other factors.

## EXPERIMENTAL

*Diets used.* The composition of the diets is shown in table 1. Diet 2 was supplemented daily with 2 drops of cod liver oil,<sup>1</sup> 6 units of a highly potent vitamin B concentrate,<sup>2</sup> and 0.5 cc. of rice bran filtrate 2<sup>3</sup> (Lepkovsky and Jukes, '35). The lard, cornstarch, yellow corn meal, wheat middlings, ground limestone, iodized salt and bone meal were ordinary commercial products. Salt mixture 2 consisted of bone ash, 38%; calcium

TABLE 1  
*Composition of the diets used*

	DIET 2 (RATS)	DIET 92 (CHICKS)	DIET 90 (TURKEYS)	DIET 60 (TURKEYS)	DIET 70 (CHICKS) <sup>a</sup>
Casein, acid washed	27 gm.	20	22	18	
Sucrose	59				
Lard	10				
Salt mixture 185 <sup>b</sup>	4				
Salt mixture 2		3	3		
Cornstarch		50	36		
Wheat bran, acid washed		20	20		
Yellow corn meal				53	
Wheat middlings				22	
Liver filtrate		4 cc.		4 cc.	
Rice bran filtrate 1			15 cc.		
Ground limestone				1	
Calcium carbonate, precipitated			1		
Sodium chloride, iodized				1	
Bonemeal				1	
Cod liver oil, medicinal	*	2	3	2	
Hexane extract of alfalfa meal, 1 cc. $\approx$ 2 gm.		0.5 cc.	0.5 cc.	0.5 cc.	
Tap water		4			
Vitamin B concentrate 1 <sup>c</sup>		0.3 cc.			
Vitamin B concentrate 2 <sup>d</sup>			0.1 cc.		

\* Lepkovsky and Jukes ('35).

<sup>b</sup> McCollum and Simmonds ('18).

<sup>c</sup> Assayed with chicks, and fed at a level to provide a 50% excess.

<sup>d</sup> Assayed with chicks, and fed at three times the level necessary for chicks.

\* See below.

<sup>1</sup> The cod liver oil was generously presented by Mead Johnson and Co., Evansville, Indiana.

<sup>2</sup> The vitamin B was kindly furnished by Dr. Elmer Stuart of the Lilly Research Laboratories, Indianapolis, Ind.

<sup>3</sup> The Vitab Products, Inc., San Francisco, furnished the rice bran concentrates, the fullers' earth adsorbate, and the vitamin G concentrates.

carbonate, 20.5; iodized salt, 23; magnesium sulfate, 7.5; potassium dihydrogen phosphate, 7.5; ferric citrate, 3.5. Wheat bran was washed twice by standing overnight in several volumes of dilute sulfuric acid at a pH of about 3, and dried at 60° to 70°. Liver filtrate and rice bran filtrate 1 have been previously described, except that the liver filtrate was treated twice with fullers' earth.<sup>4</sup> The filtrates were assayed with chicks (Lepkovsky and Jukes, '36), and fed at a level supplying at least twice the necessary amount of the 'filtrate factor' for maximal growth on the heated diet.

The alfalfa extract was prepared by Dr. H. J. Almquist by extracting alfalfa meal with hexane in a continuous extractor, and assayed by him for the anti-hemorrhagic vitamin. The level fed supplied at least twice the amount necessary to protect against hemorrhages on his basal diet (Almquist, '36).

Vitamin B concentrates 1 and 2 were prepared from a fullers' earth adsorbate<sup>5</sup> (McCollum and Simmonds, '18) of an aqueous extract of rice bran by elution with baryta, which does not dissolve vitamin G (flavin).

Crystalline vitamin G was prepared from a highly potent vitamin G concentrate<sup>5</sup> by fractional precipitation from water, and recrystallizing the active fractions from hot water.

*Care of animals.* Rats were kept on wire screen floors. They were fed on basal diet 2 for 10 days, and then the vitamin B and rice bran filtrate were added. The rats were considered ready for use after they ceased to gain in weight.

The care of chicks has been previously described (Lepkovsky and Jukes, '35). Feed consumption records were kept.

Bronze turkey poults were housed in electrically heated battery brooders with wire floors and fed the stock diet (Lepkovsky and Jukes, '35) for 12 days following hatching. They were then placed on basal diets 60 and 90 for 3 days when they were weighed and divided into two groups on each diet, one group receiving vitamin G in addition to the basal diet.

<sup>4</sup> The fullers' earth was purchased from Eimer and Amend, New York.

<sup>5</sup> See footnote 3, page 516.

*Results with rats.* On the low vitamin G diets rats grew very little or not at all. Their fur became quite generally greasy. Loss of hair occurred in greater or less degree in many rats on the head, neck, shoulders, back and flanks; sometimes small yellow scales appeared on the skin. The paws and face were generally bloody, and sometimes the bloodiness extended over the entire fur. Dried bloody or serous encrustations on the borders of the ears were very characteristic. The eyelids were generally red and swollen, and were sometimes stuck shut. Often the vibrissae were encrusted with dried blood. The feet were generally dirty with dried yellowish secretions. The tail was often dirty and sticky. One of the most characteristic symptoms resulted from gastro-intestinal disturbance causing the rat's abdomen to become distended, and diarrhea sooner or later to appear.

If vitamin G was not fed at this point, the rats slowly lost weight, the diarrhea became more severe, and death followed. Sometimes rats slowly gained in weight without, however, the symptoms entirely disappearing. On close observation, such rats generally revealed coprophagy.

Addition of 10 to 20 micrograms of vitamin G daily to the diet caused an immediate and marked response in the rats. Figure 1 shows the course of improvement of a rat near death from lack of vitamin G. At the time it was first photographed (fig. 1, a) it was in a miserable and greasy condition, it was badly denuded, covered with small yellow scales, its ears and vibrissae were encrusted with dried secretions, and one eye was stuck shut. The addition of 10 micrograms daily of vitamin G for 5 days brought about a spectacular change (fig. 1, b) and the rat, while not normal in appearance, was greatly improved, and gained 13 gm. After 20 days (fig. 1, c), the rat was normal in appearance and had gained 21 gm. in weight. The rat seemed permanently injured since it gained weight but very slowly.

Ordinarily, diarrhea appeared irregularly in the vitamin G deficient rats. Diarrhea developed rather suddenly in thirteen of a group of seventeen rats; four developed a very severe

diarrhea, and nine were milder cases. All seventeen rats were then given 20 micrograms of vitamin G daily. One of the four rats with severe diarrhea died, but the other three responded immediately, and in nineteen days gained an average

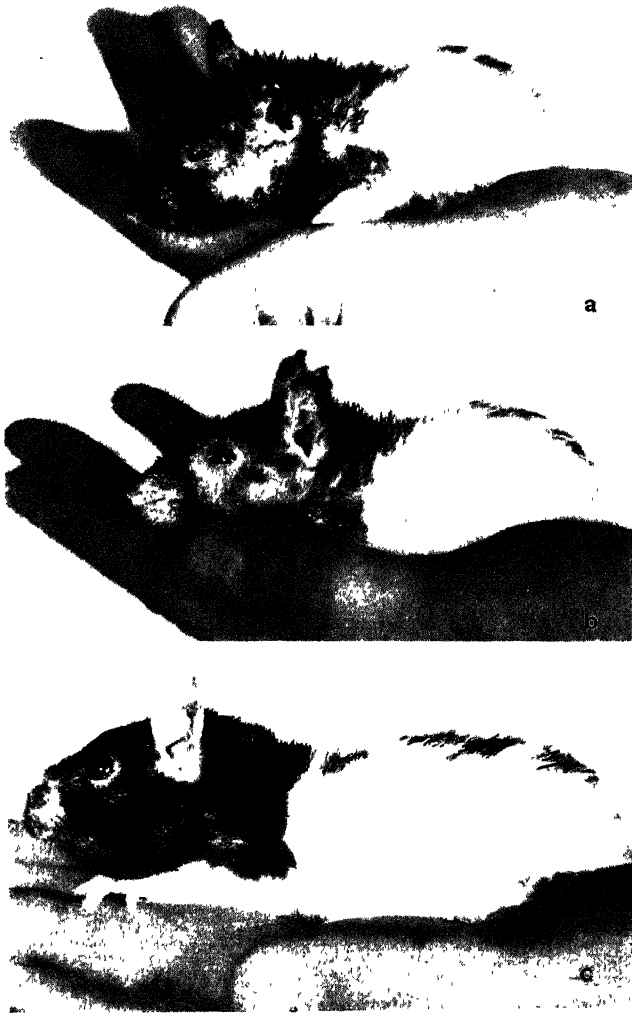


Fig. 1 Illustrating the effect of crystalline vitamin G (lactoflavin) upon a rat with acute vitamin G deficiency; a, before supplementation; b, after 5 days of feeding a daily supplement of 10 micrograms of vitamin G (lactoflavin); c, after 20 days of supplementation.

of 53 gm. in weight. While the diarrhea decreased, it did not entirely disappear, and kept recurring in a milder form. The nine rats with mild diarrhea lost the diarrhea completely

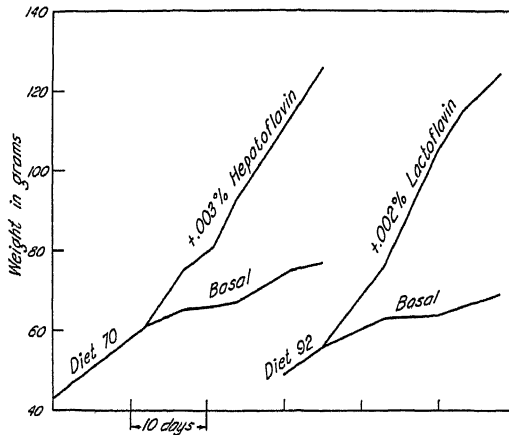


Fig. 2 Growth of chicks with and without crystalline vitamin G (flavin). Ten chicks were used in each of the four experimental groups.

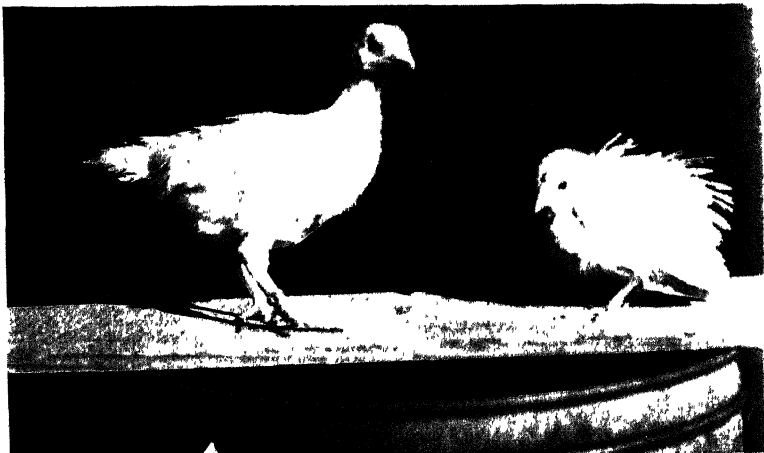


Fig. 3 Thirty-four-day-old chicks, one at right on basal diet 92, one at left on diet 92 plus 2 mg. of crystalline vitamin G (lactoflavin) per 100 gm. of diet.

and gained in 19 days an average of 46 gm. Those without diarrhea gained an average of 40 gm. in 19 days. The response in weight seemed to be in inverse ratio to the severity of the diarrhea. Other symptoms disappeared.



Fig. 4 Twenty-nine-day-old turkey after 17 days on flavin-deficient diet 60. Note the stuck eyelids and the encrustation of the mouth and nostrils.



Fig. 5 Twenty-nine-day-old turkeys; two at left after 17 days on basal diet 60, one at right on diet 60 supplemented with 2 mg. of crystalline vitamin G (lactoflavin) per 100 gm. of diet.

*Results with chicks.* Chicks on basal diet 92 (table 1) or basal diet 70 (Jukes and Lepkovsky, '35) grew very slowly and became weak and emaciated, but retained a fairly good appetite. Diarrhea developed after 8 or 10 days. There were no symptoms of dermatitis. Mortality was high after about 3 weeks. Growth of feathers did not seem to be impaired, indeed, the main wing feathers appeared to be disproportionately long in comparison with the body. The addition of vita-

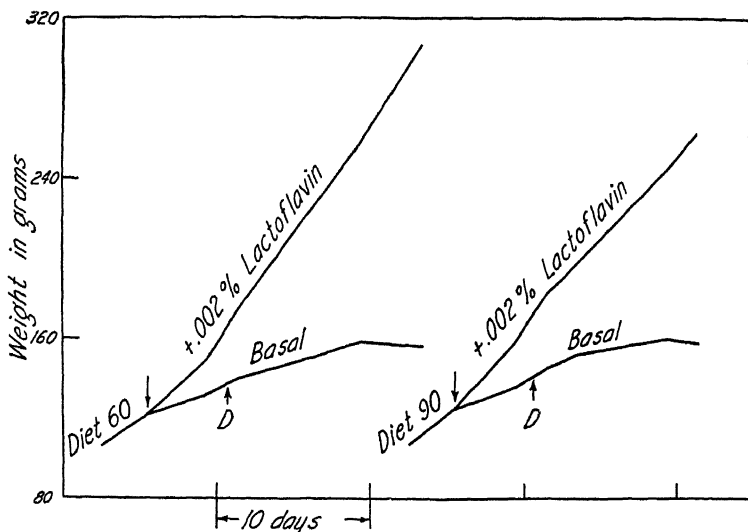


Fig. 6 Growth of turkeys with and without crystalline vitamin G (lactoflavin). Ten or eleven turkeys were used in each of the four experimental groups. 'D' indicates the appearance of dermatitis.

min G caused a marked response in growth, and in the efficiency with which food was utilized for growth. The birds receiving vitamin G appeared fairly healthy, but they did not grow so fast as chicks on a complete 'natural' diet. They did not develop diarrhea. Figures 2 and 3 illustrate the effect of the addition of vitamin G.

*Results with turkeys.* Dermatitis started to appear in turkeys after 8 days on either diet 60, consisting of natural foodstuffs, or diet 90, which was a more purified diet. The external symptoms, illustrated in figures 4 and 5, were similar



to those of chick dermatitis as first described by Ringrose, Norris and Heuser ('31), but the vent also became encrusted, inflamed and excoriated. Growth slowed up and completely ceased by the seventeenth day. Deaths started to occur on the twenty-first day. Supplementation with 2 mg. of vitamin G (lactoflavin) per 100 gm. of diet prevented the appearance of these symptoms during the experimental period of 3 weeks. Growth was rapid, and there was no mortality. The effect of vitamin G is illustrated in figures 5 and 6. Records of the feed consumption showed, as in the case of the chicks, a greater efficiency in the feed utilization. The birds on basal diet 60 consumed 7.0 gm. of feed per 100 gm. of live weight, and gained 0.20 gm. per gram of feed consumed. With the addition of vitamin G, 9.4 gm. of feed were consumed per 100 gm. of live weight, but the gain per gram of feed was increased to 0.53 gm. Similar results were obtained with diet 90.

#### DISCUSSION

There are good grounds to believe that many of the previous investigations of vitamin G deficiency were concerned with a multiple deficiency (Lepkovsky, Jukes and Krause, '36). It is thought that the experiments reported in the present communication deal more nearly with a single deficiency, since the high potency of the crystalline vitamin G (lactoflavin) employed indicates that it closely approximated to purity. Vitamin G (lactoflavin) restored rats to normal as far as could be judged by the short experimental periods employed. The symptoms developing in the deficient rats were more or less uniform. Coprophagy was a disturbing factor and tended to obscure the results. It seems clear that a dermatitis of a definite type appeared which could be cured with vitamin G. It is distinctly different from the dermatitis described by György ('35) for 'vitamin B<sub>6</sub>' deficiency. The dermatitis had much in common with that described by Goldberger and Lillie ('26). There were, however, some striking differences. The ears, while covered with a "yellowish incrustation of dried serum," were not 'reddened' nor 'thickened' nor, 'in healing'

did it leave "the skin of the pinna with a polished, glistening, and somewhat parchmentlike appearance." No "linear fissuring or ulceration at the angles of the mouth" were noticed. Other observations in this laboratory indicate Goldberger and Lillie were not working with a single deficiency (Lepkovsky, Jukes and Krause, '36). In contrast to these findings are those of Dimick, Smith and Davis ('36) who found no skin symptoms in rats nor any appreciable diarrhea. Dimick and co-workers, however, used different basal diets and vitamin B supplements from those reported in this investigation.

The contrast in symptoms between chicks and turkeys is very striking in view of the fact that these species belong to the same family of birds (Evans, 1899). Furthermore, the dermatitis developed by turkeys deficient in vitamin G is similar to the dermatitis developed by chicks suffering from a deficiency of an entirely different factor (Lepkovsky and Jukes, '35). This factor (the 'filtrate factor') was included at a high level in basal diets 92, 90 and 60. If turkeys are used as a criterion, vitamin G is the 'anti-dermatitis vitamin,' but if chicks are being studied, the title of 'anti-dermatitis vitamin' belongs to the filtrate-factor (Lepkovsky and Jukes, '36). This serves to caution against the too ready identification of dermatitis with specific deficiencies.

The purified diets 92 and 90 were not complete but the observations made over the period of study, with and without vitamin G, probably represent observations definitely related to this vitamin.

#### SUMMARY

1. Diets deficient in vitamin G (flavin), but which supplied adequate amounts of the other members of the vitamin B complex, were devised for rats, chicks and turkey poults. Vitamin G deficiency was studied by means of these diets, which permitted approximately normal growth and appearance when supplemented with small amounts of crystalline vitamin G (flavin).

2. Dermatitis and other symptoms associated with vitamin G deficiency in rats are described.

3. The symptoms of vitamin G deficiency in chicks were slow growth, diarrhea and emaciation, without dermatitis.

4. An acute dermatitis developed in turkey poults due to vitamin G deficiency, in spite of the fact that the diet was amply supplied with the 'filtrate factor,' which prevents dermatitis in the chick (Lepkovsky and Jukes, '36). The dermatitis was completely prevented by vitamin G (lactoflavin).

5. Vitamin G deficiency in chicks and turkeys appeared to reduce the efficiency of utilization of food more conspicuously than the appetite.

6. The results are discussed briefly in relation to previous investigations of the vitamin B complex.

The assistance of K. K. Miya in the care of the chicks and turkeys is gratefully acknowledged.

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## THE ANTIRACHITIC VALUE OF IRRADIATED YEAST IN INFANTS

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ONE PLATE (FOUR FIGURES)

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The earliest report on the efficacy of irradiated yeast as a source of vitamin D for humans was made by Hess ('27). He found that the daily administration of 0.5 to 1.0 gm. of irradiated yeast to rachitic infants brought about a definite curative process. The vitamin D potency of the yeast was not mentioned. In 1930, Kon and Mayzner found that the daily administration of 0.75 gm. of irradiated yeast to rachitic infants brought about a complete disappearance of the symptoms in the course of 6 to 8 weeks, with a rapid rise in the inorganic blood phosphorus and x-ray evidence of advanced healing. In a subsequent article Kon ('31) reported that the yeast used contained 1 Steenbock unit of vitamin D in 1 mg. This means that the 0.75 gm. of the irradiated yeast which was administered daily to the rachitic infants contained 2025 International vitamin D units. Compere, Porter and Roberts in 1935 reported the curative effect on rachitic infants of the daily administration of 6755 International vitamin D units in the form of irradiated yeast.

During the past 3 years we have found that comparatively small amounts of vitamin D in various forms prevented the development of rickets in infants (Drake, '36). The present paper is a report on the effect of the daily administration of approximately 500 International vitamin D units as irradiated

yeast in the prevention and cure of rickets. For ease of administration the irradiated yeast was mixed with farina.<sup>1</sup> The administration twice daily of 2 heaping tablespoons of the cooked farina containing the irradiated yeast furnished 500 International vitamin D units.

#### PREVENTIVE TESTS

For the prevention of rickets sixty-nine infants were observed over a period of 5 winter months. They represented a cross section of the infant population of Toronto and ranged in age from 4 to 7 months at the initial examination in October and November (table 1). The infants were examined clinically and by x-ray for the presence or absence of rickets according to the procedure as previously reported Drake, Tisdall and

TABLE 1  
*Ages of infants in months at time of initial examination*

GROUP	UNDER 1 MONTH OF AGE	1 MONTH OF AGE	2 MONTHS OF AGE	3 MONTHS OF AGE	4 MONTHS OF AGE	5 MONTHS OF AGE	6 MONTHS OF AGE	7 MONTHS OF AGE	TOTAL
Irradiated yeast	0	0	0	0	13	27	23	6	69
No vitamin D	1	12	19	18	16	7	2	0	75

Brown ('34). Almost all gained in weight at or above the expected rate. In addition to cow's milk dilutions they received rusks and vegetable soup at 7 to 9 months of age. Practically none received egg yolk. Care was taken to see that no vitamin D was obtained from any source other than the irradiated yeast. We were also able to observe a series of infants receiving no vitamin D over the same period.

We are again indebted to Dr. Martha Eliot, assistant chief, Children's Bureau, Department of Labor, Washington, who kindly read the x-ray plates for us. The degree of rickets was classified by Doctor Eliot as 1-, 1, 2 and 3. In this paper we have called Doctor Eliot's group 1-, extremely slight rickets

<sup>1</sup> We are indebted to the Quaker Oats Company for supplying us with the farina containing the irradiated yeast.

(a barely perceptible change which frequently might not be noted); group 1, mild rickets (a slight but obvious rachitic change); groups 2 and 3, moderate and marked rickets (well-defined fringing and cupping at the end of the bone). In addition, the x-ray films were interpreted by Doctor Eliot as to the presence or absence of active advancement of the rachitic process. It should be noted that this last classification indicates only the state of activity of the rachitic process and does not indicate in any way the degree of rickets.

Although we have considered these infants from the standpoint of the prevention of rickets, extremely slight or mild rickets was present in some when the vitamin D therapy was instituted in the fall. Before the initial examination in the fall no vitamin D had been given by mouth although naturally

TABLE 2  
*Degree of rickets at the initial examination in autumn*

GROUP	TOTAL CASES	NO RICKETS	EXTREMELY SLIGHT RICKETS	MILD RICKETS	MODERATE AND MARKED RICKETS
Irradiated yeast	69	% 81	% 16	% 3	% 0
No vitamin D	75	88	11	1	0

most of the infants had received the vitamin D benefit of summer sunshine. In Toronto very little vitamin D effect of sunshine is obtained after the middle or end of September. In table 2 is given the degree of rickets which was found in the infants in the two groups at the initial examination. It is seen that the number of cases showing evidences of extremely slight and mild rickets is slightly higher in the irradiated yeast group.

In table 3 is shown the maximum degree of rickets observed at any time subsequent to the initial examination.

In table 4 is given the number of cases in each group showing actively advancing rickets with no evidence of healing at times of observation. As stated before, it should be noted that this classification indicates only the presence of activity in the rachitic process and does not indicate in any way the degree of rickets.

## CURATIVE TESTS

In order to determine the curative effect of irradiated yeast we gave one infant with marked rickets approximately 1000 International vitamin D units daily in the form of the cooked irradiated yeast—farina mixture. This infant was 7 months of age when the treatment was started on March 15, 1936. A comparison of figure 1, taken March 15th, with figure 2, taken April 15th, shows the curative effect as evidenced by x-ray. Although craniotables cannot be taken as evidence of the presence or absence of rickets (Drake, Tisdall and Brown, '34), it is of interest that in this case on March 15th an exceedingly

TABLE 3

*Maximum degree of rickets observed at any time subsequent to initial examination*

GROUP	TOTAL CASES	NO RICKETS	EXTREMELY SLIGHT RICKETS	MILD RICKETS	MODERATE AND MARKED RICKETS
Irradiated yeast	69	% 87	% 4	% 9	% 0
No vitamin D	75	51	16	20	13

TABLE 4

*Number of cases showing actively advancing rickets with no evidence of healing at the times of observation*

GROUP	TOTAL CASES	AUTUMN	MIDDLE OF WINTER	END OF WINTER
Irradiated yeast	69	% 6	% 4	% 0
No vitamin D	75	9	21	31

marked degree of craniotables was present, while 1 month later the craniotables had entirely disappeared.

To four other infants with marked rickets we gave approximately 500 vitamin D units daily in the form of the cooked irradiated yeast—farina mixture. In every instance marked healing resulted, as shown by x-rays taken 1 month later (figs. 3 and 4). The x-rays showing evidences of healing were taken on February 1st, April 18th, April 24th and May 14th. The weather in Toronto during the month of April 1936 was so inclement that there was no possibility of the infants receiving any antirachitic effect from exposure to sunshine or



skyshine. (Dominion Meteorological Department reports that in Toronto the month of April 1936 was the darkest April on record.)

#### DISCUSSION

The results reported in table 3 show that no infant receiving 500 International vitamin D units developed moderate or marked rickets, while 13% of the infants receiving no vitamin D developed rickets of this degree. In table 4 we see that 31% of the infants receiving no vitamin D exhibited active rickets at the concluding observation in March or April, while none of the group receiving the irradiated yeast showed any evidence of actively advancing rickets. It should be noted that the infants in the group receiving no vitamin D are somewhat younger than the infants receiving the irradiated yeast. It is recognized that these younger infants are somewhat more susceptible to rickets.

It is evident from figures 1, 2, 3 and 4 that the daily administration of approximately 1000 or 500 International vitamin D units in the form of irradiated yeast not only prevented the development of rickets but brought about in 1 month's time marked healing of severe rachitic processes.

#### SUMMARY

1. The daily administration for 5 winter months of approximately 500 International vitamin D units in the form of irradiated yeast to sixty-nine normally growing infants of British and Northern European stock prevented the development in every instance of rickets of a moderate or marked degree.

2. The daily administration of approximately 1000 International vitamin D units in the form of irradiated yeast to one infant with marked rickets and of approximately 500 units to four infants with moderate or marked rickets brought about definite healing in the course of 1 month.

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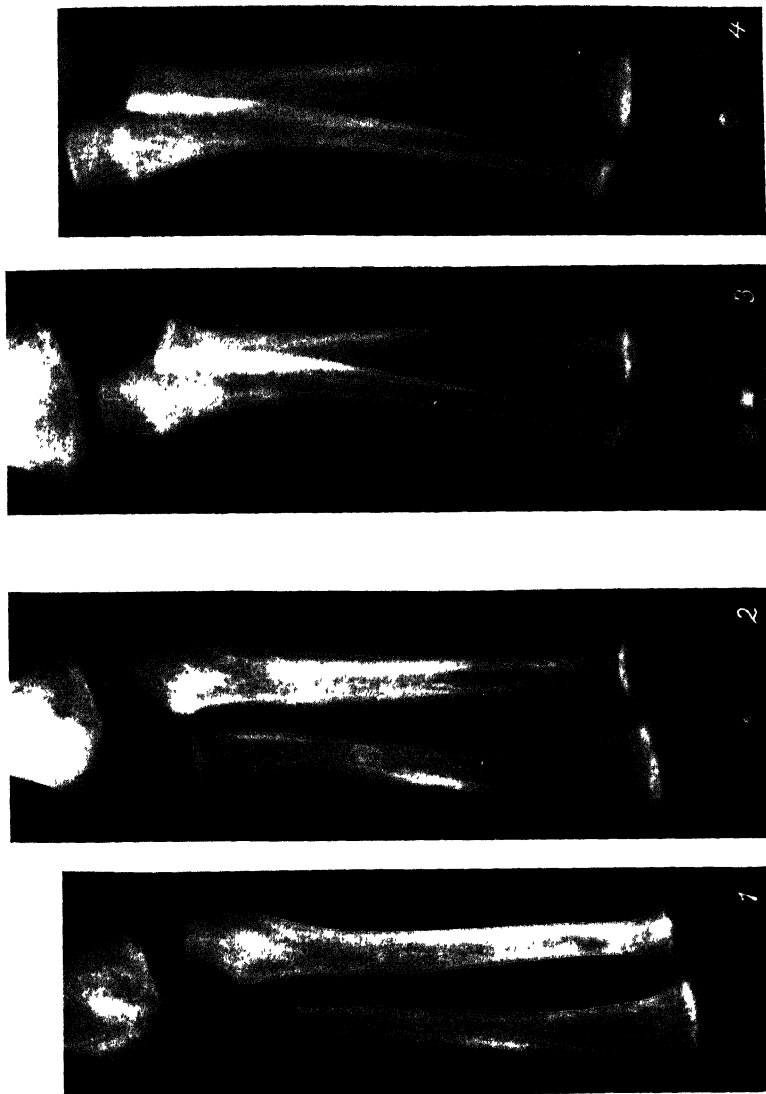
## PLATE 1

## EXPLANATION OF FIGURES

- 1 Case no. 40. X-ray of wrist of 7-month-old infant with marked rickets, March 15th. This x-ray shows evidences of marked rickets.
- 2 Case no. 40. X-ray of wrist of the same child, April 15th, after the daily administration for 1 month of 1000 International vitamin D units of irradiated yeast.
- 3 Case no. 224. X-ray of wrist of infant aged 7 months showing evidences of marked rickets, April 14th.
- 4 Case no. 224. X-ray of wrist of same infant after the daily administration for 1 month of 500 International vitamin D units of irradiated yeast.

PLATE 1

ANTIRACHITIC VALUE OF IRRADIATED YEAST  
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# DIFFERENTIATION BETWEEN VITAMIN G AND AN INSOLUBLE FACTOR PREVENTING A PELLAGRA- LIKE SYNDROME IN CHICKS<sup>1</sup>

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ONE TEXT FIGURE AND ONE PLATE

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In studies designed to throw further light on the cause of a peculiar paralysis of nutritive origin (Norris et al., '31) involving the legs and feet of chicks, Ringrose, Norris and Heuser ('31) observed a pellagra-like syndrome for the first time in this species. This pellagra-like syndrome was comparable to the pellagra in the albino rat obtained by Goldberger and Lillie ('26) and Salmon, Hays and Guerrant ('28) in so far at least as one could expect to find identical symptoms in so widely different species.

In this experimental work diets containing commercial casein, purified casein or dried raw egg white together with cereals, cod liver oil, and necessary minerals were fed. Three comparable experiments were conducted. In the first and second experiments the same supply of ingredients was used but in the third experiment a different supply was used.

In all experiments external lesions of a distinctly pellagrous character appeared in the lots receiving the egg-white diet at about 3 weeks of age. A little later the lots receiving the

<sup>1</sup> The research reported in this paper was done in partial fulfillment of the requirements for the degree of doctor of philosophy at Cornell University by A. T. Ringrose and is a part of an experiment station project on the vitamin G requirements of poultry under the direction of L. C. Norris.

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commercial-casein diet and those receiving the purified-casein diet also developed pellagrous lesions identical in appearance in so far as could be determined by detailed macroscopic examination to those which developed in the chicks on the egg-white diet. Pictures showing the striking similarity of the pellagra-like syndrome on the casein diets and the egg-white diet are presented in plate 1.

In this syndrome lesions developed at the eyes, the corners of the mouth and on the feet. The margins of the eyelids became granular and a viscous exudate was produced which frequently caused the eyelids to stick together so that the chicks could not see. Crusty scabs appeared at the corners of the mouth. These scabs gradually enlarged to involve the margins of the skin around the nostrils and underneath the lower mandible. The skin on the bottoms of the feet and between the toes gradually thickened and cornified, and small cracks and fissures appeared. These enlarged and deepened and occasionally slight hemorrhages occurred. Usually the severely affected chicks were sensitive to walking.

The average intensity of the pellagra-like syndrome of the lots of chicks on the casein diets was never as severe as that of the lots of chicks on the egg-white diet, although there were individuals on the casein diets which were just as severely affected as those on the egg-white diet.

The mortality of the chicks receiving the egg-white diet was very high in all cases, the average being 81.8% at the end of the 12-week experimental period as compared to an average of 48.7% in the chicks receiving the purified-casein diet.

The chicks in the lots receiving the egg-white diet grew at a more rapid rate at the start, than did the chicks in the lots receiving the purified-casein diet. Later, however, at the onset of the pellagra-like syndrome the growth of the chicks on the egg-white diet was slowed up sufficiently so that the growth of the chicks on the purified-casein diet finally equaled or exceeded that of the chicks on the egg-white diet. In figure 1 are presented the growth curves of these lots and also the growth curves of comparable lots used as controls in two

subsequent experiments. However, in one of these the growth of the chicks fed the purified-casein diet never quite equaled that of the chicks fed the egg-white diet.

The biometrical significance of the mean growth difference at 3 weeks of age, the approximate age of the greatest difference, between the average of the mean weights of the lots fed the purified-casein diet and the lots fed the egg-white diet in all experiments was 13.5 times greater than the standard error of the difference.<sup>3</sup> This difference in growth was, therefore, highly significant.

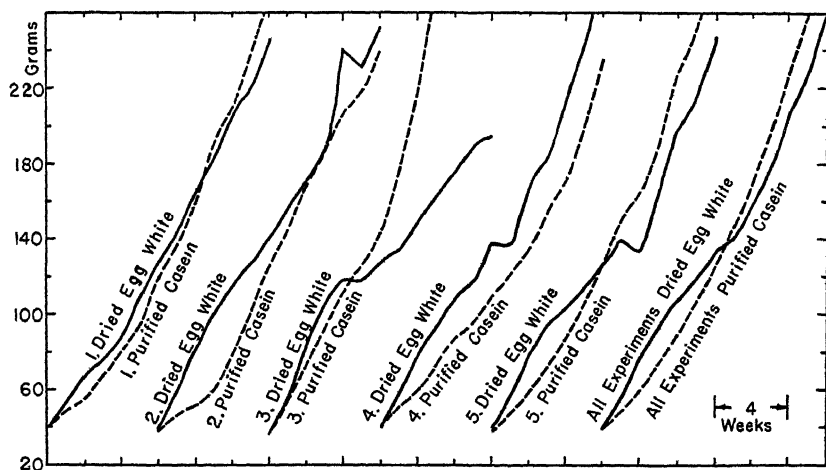


Fig. 1 Difference in growth response between the chicks fed the egg-white diet and those fed the purified-casein diet.

It was evident, then, that the egg-white diet was greatly deficient in a pellagra-preventing factor but contained a fairly large amount of a growth-promoting factor while the purified-casein diet was nearly adequate in a pellagra-preventing factor but very deficient in a growth-promoting factor. In view of these results it was felt desirable to make a further study of the possibility of the existence of a factor required to prevent the occurrence of the pellagra-like syndrome on the egg-white diet and of a factor required to promote normal growth on

<sup>3</sup> Under the conditions of these experiments a difference 2.5 times greater than its standard error is significant.

the purified-casein diet. The growth-promoting factor was vitamin G ( $B_2$ ) in the sense of the term generally accepted at the time this investigation was undertaken, since Ringrose, Norris and Heuser ('31) found that autoclaved yeast promoted growth and prevented the development of pellagra-like lesions in chicks, fed a cereal, casein diet deficient in this vitamin. Whether or not the pellagra-preventing factor had been considered a part of a so-called vitamin G complex up to this time could not be determined from this evidence.

A syndrome similar to the pellagra-like syndrome which developed in chicks fed the raw egg-white diet has been obtained in the albino rat by other investigators. Several of these have advanced a different hypothesis to explain their results than that of a nutritional deficiency disease as indicated by the results presented here. Among these is Boas ('27) who reported, that a diet containing dried egg white as the sole source of protein was unsatisfactory for growth in young rats and that on such a diet a characteristic syndrome, the chief symptoms of which were dermatitis, baldness and spastic gait, developed. Also, when the egg white was coagulated before feeding the syndrome no longer developed. From these results, Boas ('27) suggested two alternative explanations; the development of some toxic substance during the drying of egg white, capable of neutralization by certain protective foods or the destruction of some hitherto unrecognized dietary essential when drying the egg white before coagulation. However, neither explanation was entirely satisfactory.

Findlay and Stern ('29) confirmed the finding of Boas that dried egg white resulted in nervousness and cutaneous symptoms but their investigations of the pathological changes occurring led them to believe that the condition in rats was similar to Swift's disease in children. The fact that the same disease was produced in suckling rats whose mothers received dried egg white caused them to postulate the deficiency of a dietetic factor as opposed to the presence of a toxic substance.



Boas-Fixsen ('31) after further work came to the conclusion, however, that the weight of evidence favored the explanation that dried egg white contained some toxic substance created during the drying process and opposed the theory that the nutritive disorder was due to a deficiency as believed by Findlay and Stern.

Parsons ('31) pointed out that, of the diets rich in protein studied, the one high in egg white was the only one exhibiting markedly unfavorable physiological effects on rats in striking contrast to results obtained with diets high in casein, dried liver, beef powder and egg yolk. She favored the theory of a toxic principle rendered innocuous by the presence of a protective factor or by cooking of the egg white. On rations containing 66% of dried or fresh egg white, supplemented with 20% of dried yeast, 20% of dried liver was required to prevent the development or to cure the characteristic symptoms in rats.

Boas-Fixsen and Parsons both recognized that the symptoms which they obtained on egg-white diets were similar to pellagra but did not feel that a deficiency theory or lack of a dietary essential such as vitamin G satisfactorily explained their results. Their adherence to the toxic theory was prompted primarily by the fact that cooked egg white no longer exhibited abnormal physiological effects. Moreover, Parsons reported a decrease in rate of growth with increase in quantity of egg white in the diet and used this evidence in support of the toxic theory. Boas found that a reduction of dried egg white to one-half the usual amount did not decrease the severity of the symptoms produced.

In spite of the reports of Boas-Fixsen and of Parsons it did not seem reasonable that egg white contained a toxic factor which could be destroyed by cooking. It seemed more probable that the egg-white diet was lacking in some dietary essential which was supplied by other food substances as held by Findlay and Stern in view of the apparent identity of the pellagra-like syndrome which developed on the egg-white and purified-casein diets used in this investigation.

The fact that egg white is almost the only source of protein used by the developing chick embryo (Needham, '31) not only gives support to this hypothesis but also suggests a more probable explanation for the failure of the pellagra-like syndrome to develop on cooked egg white than the toxicity theory of Boas-Fixsen and of Parsons. This is that, during the coagulation of egg white, there is formed or released from egg white a factor which prevents the pellagra-like syndrome and that the pellagra-preventing factor cannot be formed or released from raw egg white by the normal digestive processes of the animal or chick whereas in the normal metabolic processes of the developing chick embryo this occurs.

#### EXPERIMENTAL PROCEDURE

In these studies single comb White Leghorn chicks of the Cornell strain were used. In general the lots contained twenty chicks each. The chicks in each lot were individually weighed, identified by wing bands and placed in the experimental pens approximately 24 hours after hatching. Thereafter they were weighed individually every week for the duration of the 8-week experimental period. Brooders and pens were equipped with screen floors in order to prevent coprophagy. Feed and water were kept continuously before the chicks throughout the duration of each experiment. A weekly record of feed consumption was kept.

The diets were so composed as to supply protein at a level of 25% of the dry matter. This corresponds to approximately 22% air dry basis. Such a level of protein is greater than that found by this laboratory (Norris and Heuser, '30) to be required to supply a sufficient amount for normal growth. Thus, the possibility of the quantity of protein becoming a limiting factor in the experimental diets was removed.

In order to determine the severity of the pellagra-like syndrome which developed on the various diets, weekly observations of each chick were made at the time of weighing. These observations were made by the same observer and were based on a consideration of the lesions at the corners of the

mouth, at the eyes and on the feet. These, when present, were recorded by a numerical rating of 1, 2 or 3, meaning respectively slight, medium or severe. The lesions at the mouth were designated  $M_1$ ,  $M_2$  or  $M_3$ ; at the eyes  $E_1$ ,  $E_2$  or  $E_3$  and on the feet  $F_1$ ,  $F_2$  or  $F_3$ . This gave a possible maximum degree of severity of 9 when the three symptoms were each rated as 3. The total degree of severity of the pellagra-like syndrome for any week was determined by adding up the sub-numbers for all the afflicted chicks in a group and dividing by the total number of chicks in that group times 9. This figure represented the maximum severity possible. When multiplied by 100, an expression of the percentage degree of severity of the pellagra-like syndrome for any group of chicks for any week was obtained. The necessary calculations are represented by the following formula:

$$\frac{M_n + E_n + F_n}{C \times 9} \times 100 = \% \text{ degree of pellagra-like syndrome}$$

Where  $n$  = observed severity of pellagrous lesion at  $M$  (mouth), at  $E$  (eyes) and  $F$  (feet).  $C$  = number of chicks per lot.

Preliminary experiments were undertaken for the purpose of finding suitable supplements to the egg-white diet which would prevent the development of the pellagra-like syndrome and give normal growth. A typical control diet containing egg white is given below:

	%
Yellow corn meal	58.75
Wheat flour middlings	20.00
Egg white	16.75
Cod liver oil	1.00
Steamed bone meal	2.00
Limestone	1.00
Salt (iodized)	0.50
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	100.00

The dried egg white<sup>4</sup> used in this work was a commercial dried undenatured product which was finely ground. It received no special treatment before being incorporated into the diet.

<sup>4</sup> Obtained from Jaburgh Bros., New York City.

The results of these experiments showed that dried yeast, autoclaved yeast or dried egg yolk fed at a 10% level in the egg-white diet did not prevent the syndrome. However, the feeding of 10% of dried liver, prepared from frozen pigs' liver by moderate cooking, gave normal growth and entirely prevented the pellagra-like syndrome throughout the experimental period of 8 weeks. In a subsequent experiment it was found that 7.5% of dried liver was sufficient for this purpose. Therefore, dried pork liver was used at the 7.5% level in further studies on this problem.

The first experiment was undertaken in order to find, if possible, a method or methods whereby the factor in liver which prevents the pellagra-like syndrome could be destroyed. Four different treatments were applied to the liver as follows: Wet autoclaving for 6 hours and 30 hours, alkaline autoclaving for 6 hours and dry autoclaving for 30 hours. In the preparation of wet-autoclaved liver, dried liver meal was mixed with water and allowed to soak until the whole was a jelly-like mass. This mixture was then placed in the autoclave in large pans 2 inches deep. The pressure in the autoclave was maintained at 15 pounds for 6 hours when part of the liver was removed, the remainder being autoclaved at 15 pounds until 30 hours had elapsed. The two lots, autoclaved wet, were then dried in a stream of air and ground. The alkaline autoclaved liver was prepared by mixing dried liver with water, until it could be readily stirred with a wooden paddle when a sodium hydroxide solution (approximately 25%) was added until pH 9 was reached as determined by the hydrogen electrode. The alkalized liver was allowed to stand over night during which time practically no reduction in pH occurred. Then it was placed in pans in the autoclave and autoclaved for 6 hours at 15 pounds' pressure. During the autoclaving the pH dropped to about 7.5. The material was then brought to pH 4.5 to 5.0, dried in a stream of air and ground. Dry-autoclaved liver was prepared by placing dried liver without previous addition of water in pans in the autoclave and autoclaving for 30 hours at 15 pounds pressure. This material absorbed so little

water during this process that subsequent drying and grinding was unnecessary.

These different liver preparations were then fed at a level of 7.5% by including them in the egg-white diet in such a way as to maintain the protein level at 25% dry basis. The results are presented in table 1. The control egg-white diet gave the usual results in the way of poor growth, high mortality and severe pellagra-like lesions. The egg-white diet containing 7.5% dried liver which served as the positive control gave normal growth with no mortality and no pellagra-like lesions.

TABLE 1

*Treatment of liver to destroy its pellagra-preventing properties when combined with the egg-white diet*

EXPERIMENT NO.	LOT	GROWTH, WEEKS 8	MORTALITY, WEEKS 8	PELLAGRA, WEEKS		
				4	6	8
8	EW (egg white)	gm. 268	% 35	% 57	% 56	% 55
8	EW + 7.5% liver	666	..	..	..	..
8	EW + 7.5% liver A <sup>1</sup> wet 6 hours	568	..	..	..	..
8	EW + 7.5% liver A pH 9 6 hours	577	..	..	..	..
8	EW + 7.5% liver A wet 30 hours	417	5	..	..	..
8	EW + 7.5% liver A dry 30 hours	296	15	23	33	33
7	EW + 7.5% liver A dry 30 hours	362	32	60	52	28

<sup>1</sup> A = autoclaved.

The results from the lots fed egg-white diets containing liver wet autoclaved for 6 and 30 hours showed that neither of these treatments destroyed the pellagra-preventing factor in liver. However, the growth was somewhat less than that on the untreated liver, particularly on the liver which was autoclaved wet for 30 hours.

The alkaline autoclaving at pH 9 also did not destroy the pellagra-preventing factor of liver since no pellagric lesions occurred. Again the growth response in this lot was somewhat below that of the lot on the untreated liver.

The results obtained by feeding liver dry autoclaved for 30 hours showed that this treatment destroyed the pellagra-preventing factor in liver so completely that the pellagra-like

syndrome was as severe as on the control diet. The results confirmed those of a preliminary experiment in which there was one lot fed the egg-white diet containing liver dry autoclaved for 30 hours. These results are presented in table 1 for comparison with those of the present experiment. The pellagra-preventing factor therefore was unstable to dry autoclaving. These results on dry autoclaving together with the appearance of a paper by Kline et al. ('32) in which it was reported that dry heat treatment destroyed a pellagra-preventing factor required by chicks led to the adoption of the latter treatment in the next experiment.

This experiment was undertaken to obtain a separation of the factor which prevented the pellagra-like syndrome from the growth-promoting factor. In this experiment the egg-white diet was used to determine the presence of the pellagra-preventing factor and the purified-casein diet was used to determine the presence of the growth-promoting factor. It has already been pointed out that the egg-white diet was well supplied with the growth-promoting factor and that the purified-casein diet was low in this factor. The purified-casein diet used in this experiment is given below:

	%
Yellow corn meal	61.50
Wheat flour middlings	20.00
Purified casein	14.00
Cod liver oil	1.00
Steamed bone meal	1.00
Limestone	2.00
Salt (iodized)	0.50
	<hr/>
	100.00

Dried liver was used as a source of both the growth-promoting and pellagra-preventing factors. Three different treatments were given this material in attempting to separate the factors. These were heating in a dry atmosphere, alkaline autoclaving and extraction.

The heated liver was prepared by heating the dried liver in thin layers in a dry atmosphere for 50 hours at 120°C. The autoclaved liver was prepared by treating the dried liver

with a sodium hydroxide solution with stirring until the pH was 11 as determined with a hydrogen electrode. The mixture was then allowed to stand overnight and, if the pH had fallen, (never fell below pH 10.5) more alkali was added to bring it back to pH 11 before placing in the autoclave. After the material had been autoclaved at 15 pounds pressure for 6 hours it was removed from the autoclave and a pH determination made. It was found that the pH dropped during the autoclaving to pH 9.4. Sufficient hydrochloric acid was then added to bring the pH to 4.5 to 5.0, when the liquid mass was dried and ground.

An extract of dried liver was prepared by using an ethyl alcohol-water mixture containing 20% alcohol by weight. The liver was first defatted with ether, then air-dried and the equivalent of 1 kilo. dried liver (approximately 850 gm. defatted liver) was treated with 2 liters of the alcohol-water mixture, allowed to stand for an hour with frequent stirring and then filtered. The filtrate was then brought to a temperature of 80°C. to coagulate any protein remaining and filtered a second time. This extraction was repeated twelve times, the filtrate becoming practically colorless at the end of that time. The combined filtrates (approximately 24 liters) were concentrated under a vacuum of 650 mm. of Hg and the volume adjusted so that 1 cc. was equivalent to 1 gm. of the original dried liver. After extraction the residue was dried and ground.

This experiment contained twelve lots of chicks, six of which received the egg-white diet while the other six received the purified-casein diet. Dried liver, liver extract, liver residue, heated liver and alkaline autoclaved liver were used as supplements for both diets. The lots on the egg-white diet received the liver supplements at a level equivalent to 7.5%. Since preliminary results had shown that 2.5% of dried liver promoted normal growth in chicks fed the purified-casein diet the lots on this diet received the liver supplements at a level equivalent to 2.5% of dried liver.

The summary of the results of this experiment are presented in table 2 together with those of a duplicate experiment in which a different supply of egg white, purified casein and dried liver were used. The various supplements were prepared in the same way and fed at the same levels as in the original experiment.

The chicks on the egg-white control diet responded as usual with more rapid growth at the start than those on the purified-casein control diet but slowed up at the onset of the syndrome

TABLE 2

*Differentiation of the growth promoting factor from the pellagra-preventing factor*

EXPERI- MENT NO.	LOT	GROWTH, WEEKS 8	MORTALITY, WEEKS 8	PELLAGRA, WEEKS		
				4	6	8
		gm.	%	%	%	%
13	EW (egg white)	206	30	41	56	53
14	EW (egg white)	227	45	61	67	64
13	EW + 7.5% liver	648	5	..	..	..
14	EW + 7.5% liver	646	15	..	..	..
13	EW + liver extract <sup>1</sup>	286	40	35	68	55
14	EW + liver extract <sup>1</sup>	219	70	62	80	78
13	EW + liver residue <sup>1</sup>	578	5	3	..	..
14	EW + liver residue <sup>1</sup>	604	15	13	8	..
13	EW + heated liver <sup>1, 3</sup>	356	55	21	37	44
14	EW + heated liver <sup>1, 3</sup>	363	55	52	51	47
13	EW + A liver <sup>1, 4</sup>	342	30	22	32	48
14	EW + A liver <sup>1, 4</sup>	347	20	47	50	48
13	PC (purified casein)	242	40	..	..	..
14	PC (purified casein)	357	15	..	..	..
13	PC + 2.5% liver	703	..	..	..	..
14	PC + 2.5% liver	652	10	..	..	..
13	PC + liver extract <sup>2</sup>	567	..	..	..	..
14	PC + liver extract <sup>2</sup>	621	10	..	..	..
13	PC + liver residue <sup>2</sup>	402	5	..	..	..
14	PC + liver residue <sup>2</sup>	485	10	..	..	..
13	PC + heated liver <sup>2, 3</sup>	586	10	..	..	..
14	PC + heated liver <sup>2, 3</sup>	665	10	..	..	..
13	PC + A liver <sup>2, 4</sup>	388	10	..	..	..
14	PC + A liver <sup>2, 4</sup>	631	10	..	..	..

<sup>1</sup> Equivalent to 7.5% liver.

<sup>2</sup> Equivalent to 2.5% liver.

<sup>3</sup> Liver heated for 50 hours at 120°C.

<sup>4</sup> Liver autoclaved at pH 11 for 6 hours at 15 pounds.



so that the growth of the purified-casein chicks finally exceeded that of the egg-white chicks. Severe pellagra-like lesions developed in the chicks on the egg-white diet causing a higher average mortality than that occurring in the chicks on the purified-casein diet in which none of the syndrome developed.

The addition of dried liver to the egg-white diet caused a normal rate of growth to take place and the development of the syndrome was prevented. The addition of dried liver to the purified-casein diet also caused normal growth.

Whenever liver extract displaced dried liver in the egg-white diet the pellagra-like syndrome was severe and a high mortality occurred. Growth was just as poor as on the control diet. The lots receiving the extracted residue, however, showed practically no symptoms of the syndrome and little mortality. Growth was nearly equal to that of the unextracted liver. On the other hand, whenever liver extract displaced dried liver in the purified-casein diet, practically normal growth resulted whereas the liver residue gave growth only slightly better than the purified-casein control. The mortality was small in both cases.

The chicks of the two lots fed the egg-white diet containing liver heated in a dry atmosphere for 50 hours at 120°C. developed severe pellagra-like lesions, the mortality was high and growth was no better than that of the control lots. This showed that dry heat treatment destroyed practically all of the pellagra-preventing factor of liver. These results are in agreement with those presented in table 1 which showed that liver autoclaved dry for 30 hours also destroyed the pellagra-preventing factor. In striking contrast to these results the chicks of the two lots fed the purified-casein diet containing liver heated in a dry atmosphere showed that this liver induced a rapid rate of growth with no mortality and none of the pellagra-like syndrome.

Alkaline autoclaving at pH 11 also destroyed the pellagra-preventing factor of liver as shown by the results from those lots which received the egg-white diet containing alkaline

autoclaved liver. The pellagra-like syndrome was practically as severe, the mortality as high and the growth as poor as on the egg-white control lots or those receiving heated liver or liver extract. In the first experiment on the purified-casein diet the results of alkaline autoclaving liver at pH 11 showed that the growth-promoting factor of liver was also sensitive to this treatment, but in the second experiment little destruction of this factor took place. In both cases the liver was at pH 11.0 to 11.3 when put into the autoclave and was at pH 9.4 when removed. However, a different supply of dried liver was used in each experiment. Possibly then the growth-promoting factor was afforded greater protection by one supply of liver than by the other.

The results of these duplicate experiments show, therefore, that the factor which prevented the pellagra-like syndrome on the egg-white diet is different from the one which promoted growth on the purified-casein diet, since the pellagra-preventing factor was insoluble in the alcohol-water mixture, was destroyed by heating in a dry atmosphere and was somewhat less sensitive to alkaline autoclaving at pH 11.

These results are in accord with the findings of Boas-Fixsen ('31) that the factor which prevents the pellagra-like syndrome is insoluble in water. She showed that casein extracted with dilute acetic acid solution and yeast extracted with boiling dilute acetic acid solution still contained the protective factor.

The results of Parsons and Lease ('34) published after the completion of the work presented in this report showed that the factor in liver which prevents or cures the pellagra-like syndrome in rats developed by feeding egg-white diets is contained in the solid liver residue remaining from the preparation of Eli Lilly and Company liver extract no. 343 and is not present in the extract and that it is destroyed by dry heating liver for 6 days at 100°C. Later Lease and Parsons ('34) in work with chicks fed an egg-white diet offered further confirmation of the fact that the factor concerned is not removed from liver by extraction but remains in the extracted residue.

Salmon and Goodman ('34) showed likewise that well-developed cases of the syndrome in rats were apparently cured by brewer's yeast and the extracted residue of brewer's yeast but not by the yeast extract or by extracted casein. The casein may have been fed, however, at too low levels, since Gorter ('35) has shown that purified casein contains the factor required to prevent the egg-white syndrome in rats.

In experiments on the stability of the pellagra-preventing factor, not reported here, studies of the effect of autoclaving egg white were made. The autoclaving was carried out by placing dried egg white at its natural pH (5.9 to 6.0) in pans in the autoclave at a pressure of 15 pounds for 6 hours, after

TABLE 3

*Effect of autoclaving on the pellagra-developing characteristics of egg white*

EXPERIMENT NO.	LOT	GROWTH, WEEKS 8	MORTALITY, WEEKS 8	PELLAGRA, WEEKS		
				4	6	8
6	EW (egg white)	gm. 151	% 76	% 51	% 49	% 48
17	EW	201	35	63	74	83
6	EW, autoclaved 6 hours	356	8	..	..	..
17	EW, autoclaved 6 hours	449	30	..	..	..
7	EW, autoclaved 6 hours pH 9	413	..	..	..	..

which time the material was removed from the autoclave, dried and ground. The alkaline autoclaved egg white was prepared by adding sodium hydroxide solution (approximately 10%) to the egg white powder until pH 9 was reached as determined by the hydrogen electrode. This material was then autoclaved for 6 hours at 15 pounds pressure, dried and ground. The pH after autoclaving was found to be 7.5. Two lots of chicks were fed the regular egg-white diet as a control. Two other lots were fed autoclaved egg white in place of the regular egg white. An additional lot was fed alkaline autoclaved egg white in place of the regular egg white.

The results are presented in table 3. These results showed that after coagulation by autoclaving for 6 hours at 15 pounds

pressure at pH 5.9 to 6.0 and at pH 9 none of the pellagra-like syndrome developed. These results are in agreement with the work of Boas-Fixsen ('31) and of Parsons ('31) who finally concluded that cooking destroyed a toxic factor present in raw egg white. A more probable explanation of this, however, as pointed out in the introduction, is that the protective factor was formed or released by cooking.

#### SUMMARY

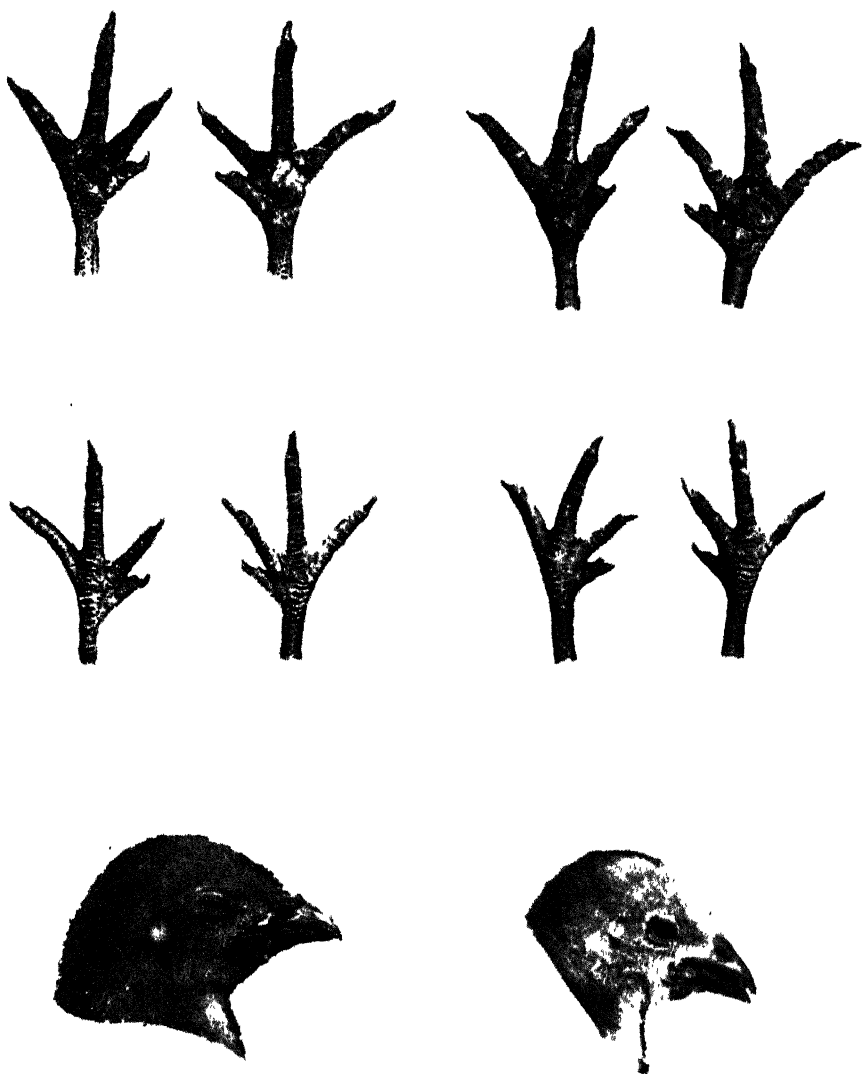
Evidence has been presented which shows that dried pork liver contains a factor required to prevent the development of a pellagra-like syndrome in chicks fed an egg-white diet and a factor required for the growth of chicks fed a purified-casein diet. This growth-promoting factor was soluble in an alcohol-water solution, was stable to heating in a dry atmosphere and relatively stable to autoclaving at pH 11. On the other hand, the pellagra-preventing factor was insoluble in an alcohol-water solution, was destroyed by heating in a dry atmosphere and by autoclaving at pH 11 but not at pH 9. This demonstrates that the pellagra-preventing factor is separate from the growth-promoting factor. The growth-promoting factor is vitamin G.

Evidence has also been presented which strongly indicates that the pellagra-like syndrome which develops in chicks fed the egg-white diet is identical with that which develops to a lesser extent in chicks fed the purified casein diet.

When egg white was autoclaved for 6 hours at 15 pounds pressure at pH 5.9 to 6.0 or pH 9, it no longer caused the development of the pellagra-like syndrome. It is believed the more probable explanation of this is that the pellagra-preventing factor is formed or released during autoclaving rather than that a toxic factor is destroyed, in view of the apparent identity of the pellagra-like syndromes and since raw egg white is almost the only source of protein used by the developing chick embryo.

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Striking similarity of the pellagra-like syndrome which developed on the egg-white and casein diets. Chick at the left fed the egg-white diet, that at the right the purified-casein diet.

# DIFFERENTIATION BETWEEN VITAMIN G AND A SOLUBLE FACTOR PREVENTING A PELLAGRA-LIKE SYNDROME IN CHICKS<sup>1</sup>

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Evidence was presented by Ringrose and Norris ('36) which showed that a factor preventing the development of a pellagra-like syndrome in chicks fed an egg-white diet was not vitamin G(B<sub>2</sub>), since this factor did not promote growth in chicks fed a purified-casein diet deficient in this vitamin and was insoluble in an alcohol-water mixture in which vitamin G is soluble. Evidence was also presented in this paper which indicated that the pellagra-like syndrome which developed on the egg-white diet was identical with that which developed to a lesser extent on the purified-casein diet. In a paper by Kline, Keenan, Elvehjem and Hart ('32) it was reported that chicks fed a casein diet heated in a dry atmosphere developed a pellagra-like syndrome similar in appearance to that obtained by Ringrose, Norris and Heuser ('31) in chicks fed either the egg-white or the purified-casein diet. In this work Ringrose, Norris and Heuser found that autoclaved yeast was effective in preventing the syndrome on the casein diet but later Ringrose and Norris ('36) found that yeast, dried or autoclaved, and liver extract were ineffective in preventing it on the egg-white diet. Dried pork liver and the residue of extracted liver on the other hand were completely effective in this respect.

<sup>1</sup> The research reported in this paper was done in partial fulfillment of the requirements for the degree of doctor of philosophy at Cornell University by A. T. Ringrose and is a part of an experiment station project on the vitamin G requirements of poultry under the direction of L. C. Norris.

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Kline et al. ('32) reported that autoclaved yeast and liver extract prevented the syndrome on their heated casein diet. Therefore, in view of the apparent identity of the pellagra-like syndrome which developed on these various diets and the discrepancy in the results on means of preventing it, studies were undertaken on the possibility that two factors were required for its prevention. In conducting these studies evidence was obtained that vitamin G was a complex consisting of two factors. The results of this discovery of which preliminary reports (Ringrose and Norris, '34; Norris and Ringrose, '35) have been made are presented in this paper together with those showing that two factors are required to prevent the development of the pellagra-like syndrome in chicks.

#### EXPERIMENTAL PROCEDURE

In these studies single comb White Leghorn chicks of the Cornell strain were used. The experimental period was of 8 weeks' duration. Each lot contained twenty day-old chicks at the start. Individual weights were taken weekly together with observations of the pellagra-like syndrome, the procedure being the same as that described by Ringrose and Norris ('36).

The heated casein diet used in this work was similar in composition to the purified-casein diet used previously by Ringrose and Norris ('36). The cornmeal, wheat flour middlings and purified casein were heated by placing in pans at a depth of approximately  $\frac{3}{4}$  inch in an oven for 144 hours at 95 to 100°C. After the heating was completed the ration was mixed in the proper proportions, using amounts corresponding to the original material and adding the cod liver oil and minerals on the regular air dry basis. After heating this diet was lacking in a pellagra-preventing factor as well as the growth-promoting factor.

The first experiment with this heated diet was to determine the amount of dried liver necessary to prevent the development of the pellagra-like syndrome and to give normal growth. Dried liver was added to this diet at levels of 0, 1.5, 3.0, 4.5, 6.0 and 7.5%. These additions were made by reducing the



amount of casein in the diet with the increase in the amount of dried liver so that the protein content of the diet was maintained at 25% moisture free basis or approximately 22% air-dry basis. The amount of cornmeal was varied with each liver addition sufficiently to make the whole equal to 100.

The results of this experiment are presented in table 1. The chicks placed on the control heated casein diet grew slowly throughout the entire 8-week experimental period. Between the second and third weeks symptoms of the pellagra-like syndrome began to develop at the corners of the mouth and on the eyelids. These lesions gradually increased in severity and the mortality at the end of the experiment was 100%.

TABLE 1

*Quantity of liver required to prevent pellagra developed on the heated purified-casein diet*

EXPERI- MENT NO.	LOT	GROWTH, WEEKS 8	MORTALITY, WEEKS 8	PELLAGRA, WEEKS		
				4	6	8
		<i>gm.</i>	<i>%</i>	<i>%</i>	<i>%</i>	<i>%</i>
15	HCD (heated casein diet)	..	100	74	100	..
15	HCD — 1.5% dried liver	394	25	16	8	..
15	HCD — 3.0% dried liver	615	15	..	...	..
15	HCD — 4.5% dried liver	637	35	..	...	..
15	HCD — 6.0% dried liver	688	5	..	...	..
15	HCD — 7.5% dried liver	690	35	..	...	..

Insofar as could be determined by a macroscopic examination, the pellagric lesions which developed on the heated casein diet were the same as those obtained previously by Ringrose, Norris and Heuser ('31) on either the egg-white or purified-casein diet, but the general character of the syndrome differed somewhat, possibly because of the presence of a multiple deficiency. When the heated casein diet was fed, the most severe lesions developed at the corners of the mouth. Granulation and sticking together of the eyelids, however, were severe and appeared with great regularity but the lesions on the feet, while they were occasionally as severe as on the egg-white diet, did not appear as regularly.

The feathers of the birds on this diet showed marked hemorrhages at the point where the feathers protrude from the follicles. The main shaft of the feathers became constricted at this point and the remaining portion of the shaft showed a lack of blood supply. These feathers eventually were lost. At about the time that definite loss of feathers was observed the skin became dry, roughened and scaly. By this time the lesions at the corners of the mouth had become large crusty scabs extending often times over the entire area under the lower mandible. The eyelids became severely granulated and the exudate caused the eyelids to stick together so that the affected chicks could not see. Quite commonly, too, lesions developed on the skin around the vent. When the general condition had become this severe death soon followed.

When the heated diet contained 1.5% of dried liver most of the pellagra-like lesions were prevented but mortality was fairly great and growth was not normal. When it contained 3.0% of dried liver, all lesions were prevented and growth almost equal to that on the higher levels of liver was attained. Because of these results it was decided to feed dried liver at the 3.0% level in further work with the heated casein diet.

The purified-casein diet fed to determine the presence of the growth-promoting factor and all supplements used with it were prepared in the same way as those used in work previously reported (Ringrose and Norris, '36). However, the quantity of dried liver fed was 1.5% instead of 2.5%, in order that this slightly sub-optimum level would make it possible to better interpret the results. Additions to both the heated casein diet and the purified-casein diet included dried liver, liver extract, liver residue and liver autoclaved at pH 11.

Two duplicate series of experiments were conducted on the purified-casein diet and the heated casein diet. The first series included experiments 15 and 17 and the second series experiments 18 and 19. The ingredients, such as cereals, purified casein and liver supplements used in the diets of the first series of experiments were from the same supply of ingredients while those used in the second series were from a new supply.

The results of the duplicate series of experiments on both the purified-casein and heated casein diets are summarized in table 2. They show that the chicks fed the purified-casein diet grew very slowly in both series and only moderate mortality

TABLE 2  
*Differentiation of the growth-promoting factor from the pellagra-preventing factor*

EXPERIMENT NO.	LOT	GROWTH, WEEKS	MORTALITY, WEEKS	PELLAGRA, WEEKS		
				4	6	8
		gm.	%	%	%	%
15	PCD (purified casein diet)	219	30	..	..	..
18	PCD (purified casein diet)	235	30	..	..	..
15	PCD + 1.5% liver	629	..	..	..	..
18	PCD + 1.5% liver	534	..	..	..	..
15	PCD + liver extract <sup>1</sup>	504	15	..	..	..
18	PCD + liver extract <sup>1</sup>	484	..	..	..	..
15	PCD + liver residue <sup>1</sup>	354	5	..	..	..
18	PCD + liver residue <sup>1</sup>	244	..	..	..	..
15	PCD + heated liver <sup>1, 3</sup>	531	..	..	..	..
18	PCD + heated liver <sup>1, 3</sup>	417	5	..	..	..
15	PCD + A liver <sup>1, 4</sup>	239	25	..	..	..
18	PCD + A liver <sup>1, 4</sup>	470	5	..	..	..
17	HCD (heated casein diet)	103	75	52	62	67
19	HCD (heated casein diet)	106	75	47	65	72
17	HCD + 3.0% liver	657	5	..	..	..
19	HCD + 3.0% liver	509	7	..	..	..
17	HCD + liver extract <sup>2</sup>	438	30	..	..	..
19	HCD + liver extract <sup>2</sup>	218	63	18	42	30
17	HCD + liver residue <sup>2</sup>	147	65	33	53	57
19	HCD + liver residue <sup>2</sup>	141	75	47	33	42
17	HCD + heated liver <sup>2, 3</sup>	131	60	44	61	58
19	HCD + heated liver <sup>2, 3</sup>	117	81	49	64	100
21	HCD + heated liver <sup>2, 5</sup>	198	61	36	43	43
17	HCD + A liver <sup>2, 4</sup>	103	70	43	61	59
19	HCD + A liver <sup>2, 4</sup>	249	25	..	..	..

<sup>1</sup> Equivalent to 1.5% liver.

<sup>2</sup> Equivalent to 3.0% liver.

<sup>3</sup> Liver heated for 50 hours at 120°C.

<sup>4</sup> Liver autoclaved at pH 11 for 6 hours at 15 pounds.

<sup>5</sup> Fed at 12% level.

occurred while on the heated casein diet growth was even slower and severe pellagra-like lesions developed accompanied by very high mortality comparable to that of the chicks fed the heated casein diet in the previous experiment.

The results showed that 1.5% dried liver added to the purified-casein diet was sufficient to promote nearly normal growth in both series of experiments. No mortality occurred. The addition of 3.0% dried liver to the heated casein diet gave comparable growth in both series of experiments. None of the pellagra-like syndrome developed and little or no mortality occurred. However, the liver used in the first series gave better growth on both diets than did that of the second one.

The results where liver extract replaced the dried liver in the purified-casein diet showed that good growth was attained by the chicks on this diet in both series of experiments, while the chicks which were fed this diet containing the extracted liver residue grew very slowly attaining an average weight only slightly better than the purified-casein control.

Similarly where liver extract replaced dried liver in the heated casein diet good growth was attained by the chicks on this diet in the first series of experiments. Mortality was not high and none of the syndrome developed. In the second series where liver extract replaced the dried liver in the heated casein diet growth was not quite as good, mortality was higher and some of the pellagra-like lesions developed. In both series the chicks which were fed the heated casein diet containing liver residue grew but little better than those on the heated casein control. Also the syndrome was practically as severe and mortality was as great. This showed that none of the pellagra-preventing factor remained in the extracted liver residue in either series. Therefore, the small amount of pellagra-like syndrome which developed in the liver-extract lot in the second series must have been due to destruction in the preparation of the extract or by some oxidative or other process after the extract was incorporated into the diet.

The results obtained in this series of experiments by feeding liver extract and liver residue show that both the pellagra-preventing factor and the growth-promoting factor are soluble in the alcohol-water mixture. This is in striking contrast to the results obtained in the comparative studies with the egg-white and purified-casein diets (Ringrose and Norris, '36)

which showed that only the growth-promoting factor was soluble in the alcohol-water mixture and that the pellagra-preventing factor remained in the extracted liver residue. It is evident, therefore, that the factor which prevents the pellagra-like syndrome developed on the egg-white diet is different from that which prevents the pellagra-like syndrome developed on the heated casein diet. This difference in solubility between these two factors is in agreement with the work with chicks of Lease and Parsons ('34) published after the completion of this work. Since the pellagic symptoms developed on these diets appeared identical, it seemed probable that two factors were concerned in the prevention of this syndrome.

Whenever liver heated in a dry atmosphere for 50 hours at 120°C. displaced the dried liver in the purified-casein diet good growth was attained but when the heated liver replaced the dried liver in the heated casein diet growth was very poor, severe pellagra-like lesions developed and high mortality occurred. An additional lot fed the heated casein diet with 12% of dried heated liver was included in a later experiment to determine whether or not this dry heat treatment destroys completely the pellagra-preventing factor of liver and that an addition of a larger quantity of the growth-promoting factor contained therein would not prevent the pellagra-like lesions. These results showed that heating liver in a dry atmosphere destroyed the pellagra-preventing factor completely, whereas, the growth-promoting factor was not materially affected by this treatment. The effect of dry heat treatment on the growth-promoting factor is in accord with results previously presented (Ringrose and Norris, '36). It is evident, also, that in spite of the difference in solubility both pellagra-preventing factors are destroyed by heating in a dry atmosphere.

The results where the purified-casein diet containing liver autoclaved at pH 11 was fed showed that the growth-promoting factor may be destroyed by this treatment since the growth in one series, although not in the other, was materially reduced

as compared to that on dried liver. When liver autoclaved at pH 11 replaced dried liver in the heated casein diet growth was poor in both series. In the lot of the first series fed alkaline autoclaved liver the pellagra-like syndrome was severe and the mortality was high but in the comparable lot of the second series no pellagic lesions developed and the mortality was less. These results showed that under certain conditions both the growth-promoting and the pellagra-preventing factors are sensitive to autoclaving at pH 11. The lack of uniformity in results where alkaline autoclaved liver was used indicated, however, that although the initial pH and the pH after autoclaving was the same in both series, either complete uniformity in the alkaline autoclaving procedure was not accomplished or that greater protection was afforded the growth-promoting and the pellagra-preventing factors by one supply of liver than by the other.

The results of this duplicate series of experiments showed that the factor which prevented the pellagra-like syndrome on the heated casein diet is different from the one which promoted growth on the purified-casein diet, since the pellagra-preventing factor was destroyed by heating in a dry atmosphere while the growth-promoting factor was relatively stable to this treatment. Both factors were sensitive to autoclaving at pH 11 and both were soluble in an alcohol-water mixture.

In the final experiment another method of separating the growth-promoting factor from the soluble pellagra-preventing factor was attempted, using both the heated casein diet and the purified-casein diet. It involved the possibility of a differential adsorption of these factors by fuller's earth. This method was based primarily upon the work of Kuhn, György and Wagner-Jauregg ('33) who obtained some evidence that two factors were required for growth and the prevention of skin lesions in rats and that one of these was adsorbed by fuller's earth while the other was not.

The pellagra-preventing and the growth-promoting factors were extracted from liver by the 20% alcohol-water mixture and treated with fuller's earth. Extract equivalent to 500 gm.

of the original dried liver was diluted to 4 liters, acidified with 150 cc. concentrated HCl (37%) and treated with 200 gm. of ~~Surrey~~ fuller's earth. The mixture was stirred constantly for 1 hour after being chilled in an ice bath to a temperature of 10°C. or below. The adsorption was carried out in the cold in order that it might be more complete. After stirring, the fuller's earth was allowed to settle and the supernatant liquid was siphoned off. The fuller's earth was then thrown out by centrifuging and washed three times with distilled water to free it of HCl. The centrifugate and washings were added to the supernatant solution which was treated a second time with 200 gm. of fuller's earth, stirred for 1 hour, and allowed to settle. The supernatant liquid was again siphoned off and the earth thrown out by centrifuging and washed free of HCl. The total 400 gm. of earth was suspended in a solution of 1200 cc. water, 600 cc. methanol and 600 cc. pyridine (practical grade) by stirring and allowed to come to room temperature for elution (summer room temperature 25 to 30°C.).

The elution of the growth-promoting factor from the fuller's earth was completed by repeated extractions with the pyridine-methanol-water solution until the supernatant liquid was colorless. This solution was then concentrated under a vacuum of 650 to 700 mm. until 2 cc. was equivalent to 1 gm. of original dried liver. This preparation, called fraction 1, adsorbable, was then ready for feeding.

The combined supernatant liquid with the washings added were placed in a 12-liter flask and NaOH was added until the solution was slightly alkaline to Congo red but acid to litmus. This solution was then concentrated under a vacuum of 650 to 700 mm. so that 2 cc. was equivalent to 1 gm. of the original dried liver. This preparation, called fraction 2, non-adsorbable, was also ready for feeding.

In the purified-casein diet the supplements were fed at a level equivalent to 2.0% of dried liver and at a level equivalent to 4.0% dried liver in the heated casein diet. Of the five lots of chicks fed the purified-casein diet, one lot served as control and the other lots received additions of liver extract, fraction 1,

adsorbable, fraction 2, non-adsorbable, and fraction 1 and 2 re-combined. Of five lots of chicks fed the heated casein diet, one lot served as control and the others received the same supplements as the lots on the purified-casein diet.

The results of this experiment are presented in table 3. The purified-casein diet as usual gave only slow growth while the heated casein diet also gave very slow growth accompanied by severe pellagra-like lesions and high mortality. When liver extract was combined with these two diets, good growth took

TABLE 3

*Differentiation of the growth-promoting factor from the pellagra-preventing factor*

EXPERIMENT NO.	LOT	GROWTH, WEEKS 8	MORTALITY, WEEKS 8	PELLAGRA, WEEKS		
				4	6	8
21	PCD (purified casein diet)	gm. 276	% 6	% ..	% ..	% ..
21	PCD + liver extract <sup>1</sup>	558	..	..	..	..
21	PCD + fraction 1 <sup>1, 3</sup>	539	..	..	..	..
21	PCD + fraction 2 <sup>1, 4</sup>	249	11	..	..	..
21	PCD + fraction 1 <sup>3</sup> and 2 <sup>1, 4</sup>	554	..	..	..	..
21	HCD (heated casein diet)	98	72	42	62	56
21	HCD + liver extract <sup>2</sup>	406	45	..	..	..
21	HCD + fraction 1 <sup>2, 3</sup>	128	47	41	65	52
21	HCD + fraction 2 <sup>2, 4</sup>	143	7	12	22	25
21	HCD + fraction 1 <sup>3</sup> and 2 <sup>2, 4</sup>	370	31	..	..	..

<sup>1</sup> Equivalent to 2.0% liver.

<sup>2</sup> Equivalent to 4.0% liver.

<sup>3</sup> Adsorbable fraction.

<sup>4</sup> Non-adsorbable fraction.

place showing again that the liver extract contained both the growth-promoting and the pellagra-preventing factors.

When fraction 1, adsorbable, was combined with the purified-casein diet, good growth equal to that of the original liver extract was obtained but when this fraction was combined with the heated casein diet growth was very poor, mortality was high and the syndrome was as severe as on the heated casein diet only. These results showed that fraction 1, adsorbable, contained the growth-promoting factor but was lacking in the pellagra-preventing factor.



When fraction 2, non-adsorbable, was combined with the purified-casein diet, growth no better than that on the purified-casein diet only was obtained. When fraction 2, non-adsorbable, was combined with the heated casein diet growth was likewise very poor and little mortality occurred. However, a small amount of the pellagra-like syndrome developed on this diet but much less than the amount which occurred when fraction 1, adsorbable, was fed. The symptoms of the syndrome which occurred were the same as far as could be determined as the symptoms in the chicks on the heated casein diet supplemented with fraction 1, adsorbable. This indicated that possibly some destruction of the pellagra-preventing factor took place in the preparation of fraction 2. These results showed that fraction 2, non-adsorbable, contained the pellagra-preventing factor but was lacking in the growth-promoting factor.

When fraction 1 and fraction 2 were re-combined and fed in the purified-casein diet, growth equal to the original liver extract and to fraction 1 alone was obtained. Likewise, when the re-combined fractions were fed with the heated casein diet, good growth took place and none of the pellagra-like lesions occurred.

Thus, additional evidence which demonstrated a definite separation of the growth-promoting factor from the pellagra-preventing factor was obtained, since the growth-promoting factor was adsorbed from liver extract by fuller's earth while the pellagra-preventing factor was not adsorbed.

These results are in agreement with those of Elvehjem and Koehn ('35) which showed that chicks required a factor for the prevention of the pellagra-like syndrome on the heated casein diet which remained in a liver extract after treatment with fuller's earth. They did not find that a separate growth-promoting factor was also required, although they showed that the growth-promoting factor (flavin) adsorbed by fuller's earth from liver extract did not prevent the occurrence of the pellagra-like syndrome. Chick, Copping and Edgar ('35) and György ('35) have reported a factor (flavin) necessary

for the normal nutrition of the rat adsorbable on fuller's earth and another factor non-adsorbable. Both factors were growth-promoting but the non-adsorbable factor was required to prevent rat pellagra while the adsorbable factor was required to prevent a mild dermatitis. Lepkovsky and Jukes ('35) have also reported a growth-promoting factor adsorbed by fuller's earth and a non-adsorbable pellagra-preventing factor required by chicks fed a heated casein diet.

In connection with the work on the heated casein diet it seemed desirable to determine whether the pellagra-preventing factor was contained in the cereal portion (cornmeal and wheat flour middlings) of the diet or in the casein or both.

TABLE 4  
*Effect of heating in a dry atmosphere on cereals and casein*

EXPERIMENT NO.	LOT	GROWTH, WEEKS 8	MORTALITY, WEEKS 8	PELLAGRA, WEEKS		
				4	6	8
12	Commercial casein + unheated cereals	gm. 313	% ...	% ..	% ..	% ..
12	Commercial casein + heated <sup>1</sup> cereals	...	100	32	49	..
12	Commercial casein heated <sup>1</sup> + unheated cereals	360	35	..	..	..
12	Commercial casein heated <sup>1</sup> + heated <sup>2</sup> cereals	...	100	24	34	..

<sup>1</sup> Heated for 144 hours at 100°C. in a dry atmosphere.

In order to do this four lots of twenty-five day-old chicks per lot were used. The first lot was fed commercial casein combined with unheated cereals, the second commercial casein with heated cereals, the third commercial casein heated with unheated cereals and the fourth commercial casein heated with heated cereals. In this experiment the heated materials were heated in a dry atmosphere at 100°C. for 144 hours, the same as the heated casein diet.

The results of this experiment are presented in table 4. The lot fed the commercial casein with unheated cereals made a fair rate of growth. The lot fed commercial casein with

heated cereals grew very slowly. The mortality was 100% before the end of the eighth week and the pellagra-like syndrome was very severe. When the heated commercial casein with unheated cereals was fed, the growth was as good as the lot which was fed unheated commercial casein with unheated cereals and no pellagic lesions developed but mortality was fairly high. Heated commercial casein with heated cereals gave very slow growth. The syndrome was severe and 100% mortality occurred by the end of the seventh week. It was evident then, that the pellagra-preventing factor was contained in the cereals of the diet and not in the casein.

In view of the fact that it was shown that the factor which prevented the pellagra-like syndrome on the casein diets was contained in the cereals (cornmeal and wheat flour middlings), the ones also used in the egg-white diet, and since the pellagra-like syndrome no longer developed when the egg white was coagulated by autoclaving (Ringrose and Norris, '36), it seemed desirable to determine the effect of heating the coagulated egg white in a dry atmosphere and feeding it in the presence of both heated and unheated cereals.

This experiment contained six lots of twenty chicks each, three of which were fed egg white combined with unheated cereals and three of which were fed egg white combined with heated cereals. Of the three lots fed unheated cereals one lot received raw egg white, the second egg white autoclaved 6 hours at 15 pounds pressure and the third egg white autoclaved 6 hours at 15 pounds pressure and then heated for 50 hours at 120°C. Likewise of the three lots fed heated cereals one lot received egg white, the second egg white autoclaved and the third egg white autoclaved and heated.

The results are presented in table 5. The lot fed egg white with unheated cereals, the regular egg white control, gave the usual results of poor growth, high mortality and severe pellagra-like lesions. With autoclaved egg white and unheated cereals the growth was fairly good, with no lesions and no mortality. With egg white autoclaved and heated plus unheated cereals growth was not as good as that on autoclaved

egg white plus unheated cereals but no pellagra-like lesions developed and mortality was slight.

On the diet containing egg white and heated cereals again growth was very poor, the pellagra-like syndrome was as severe as that on the regular egg-white diet and mortality was twice as great. Autoclaved egg white with heated cereals gave poor growth, a small amount of pellagra-like lesions and mortality twice that on the regular egg-white diet. Heated autoclaved egg white with heated cereals gave very slow growth and mortality almost twice that on the regular egg-white diet. The pellagra-like syndrome developed earlier

TABLE 5

*Effect of heating in a dry atmosphere on autoclaved egg white*

EXPERIMENT NO.	LOT	GROWTH, WEEKS 8	MORTALITY, WEEKS 8	PELLAGRA, WEEKS		
				4	6	8
		<i>gm.</i>	<i>%</i>	<i>%</i>	<i>%</i>	<i>%</i>
17	EW (egg white)	201	35	63	74	83
17	EW, A 6 hours	449	..	..	..	..
17	EW, A then heated <sup>2</sup>	330	10	..	..	..
17	EW <sup>1</sup>	153	70	54	86	87
17	EW, A 6 hours <sup>1</sup>	133	80	..	22	17
17	EW, A then heated <sup>1, 2</sup>	102	60	15	29	32

<sup>1</sup> Cereals heated 144 hours at 100°C. dry.

<sup>2</sup> Autoclaved 6 hours then heated 50 hours at 120°C. dry.

A = autoclaved.

and was slightly more severe than on the autoclaved egg white with heated cereals. The dry heat treatment when applied to the autoclaved egg white was evidently not as effective in destroying the pellagra-preventing factor as when applied to cereals and dried liver.

This experiment indicated that the factor which prevented the pellagra-like syndrome resulting from heating the cereals is contained in fairly large amounts in autoclaved egg white, since when the autoclaved egg white was fed the development of pellagic lesions was almost entirely prevented. Whether the factor concerned with preventing the pellagra developed by

heating cereals was contained in raw egg white could not be determined from these results, since in the lot fed raw egg white with heated cereals both pellagra-preventing factors may have been lacking, as it was possible that both may have been formed or released in autoclaving egg white. No differences in type of pellagic lesions could be detected in this lot which is added evidence that the two syndromes are the same. Whether one or both pellagra-preventing factors were partially destroyed by dry heating autoclaved egg white could not be determined, since dry heat treatment has been shown to destroy both factors.

In view of the fact that the pellagra-like syndrome which developed in chicks fed the egg-white diet (Ringrose and Norris, '36) appeared identical with the pellagra-like syndrome developed in chicks fed the heated casein diets it seemed evident that two factors were required to prevent the development of this syndrome, since one was insoluble in an alcohol-water mixture while the other was soluble. It has been shown that autoclaved egg white contained the factor which prevented the syndrome on the heated casein diet and Boas-Fixsen ('31) and Gorter ('35) showed that purified casein contained the factor which prevented the syndrome on the egg-white diet.

It is interesting to note in this connection that Salmon ('31) observed in work with rats fed a synthetic diet containing casein heated in a dry atmosphere for 45 hours at 130° to 140°C. a pellagra-like syndrome very similar to the pellagra-like syndrome observed by Boas ('27), Parsons ('31) and Salmon and Goodman ('34) in rats fed egg-white diets. From the evidence presented herein it appeared possible that Salmon destroyed the factor in casein which prevents the pellagra-like syndrome developed on raw egg-white diets, since, it has been shown that this factor is destroyed by dry heating and that casein lacks the factor which prevents the pellagra-like syndrome developed on the heated casein diet.

## SUMMARY

Evidence has been presented which shows that dried pork liver contains a factor required to prevent the development of a pellagra-like syndrome in chicks fed a heated casein diet and a factor required for the growth of chicks fed a purified-casein diet. Both of these factors are soluble in an alcohol-water mixture and sensitive to autoclaving at pH 11.

The pellagra-preventing factor was destroyed by heating in a dry atmosphere for 144 hours at 100°C. or 50 hours at 120°C. while the growth-promoting factor was relatively stable to this treatment. The pellagra-preventing factor was not adsorbed from liver extract by fuller's earth while the growth-promoting factor was adsorbed. Vitamin G is, therefore, a complex consisting of two components, one of which is pellagra-preventing while the other of which is essentially growth-promoting.

The pellagra-preventing factor was found to be present in the cereals only of a cereal, casein diet and not in the casein. Coagulated egg white was shown to contain the pellagra-preventing factor but whether raw egg white also contained this factor could not be determined from the results.

Further evidence was obtained which indicated the identity of the pellagra-like syndrome which develops in chicks fed the heated casein diet with that which develops in chicks fed a raw egg-white diet. Two factors were required to prevent the development of this syndrome, since one of these was soluble in an alcohol-water mixture while the other was insoluble. One was also more sensitive to autoclaving at pH 11 than the other but both were destroyed by prolonged heating in a dry atmosphere.

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# THE DISTRIBUTION AND PROPERTIES OF THE ANTI-GIZZARD-EROSION FACTOR REQUIRED BY CHICKS <sup>1</sup>

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The occurrence in chicks of a 'scurvy-like syndrome' of dietary origin was described in 1933 by Holst and Halbrook and in 1934 by Dam and Dam and Schönheyder. This condition was characterized by hemorrhage, anemia, and crater-like lesions in the gizzard lining, and was prevented according to Dam and Schönheyder by feeding hog liver, hemp seed, tomatoes or kale. The factor could be extracted from these materials with ether. In subsequent papers by Dam ('35) the ability of various dietary constituents to induce normal blood clotting time has been taken as a criterion of their potency in the antihemorrhagic factor and little has been said about the gizzard lesions. In 1935 Almquist and Stokstad published the results of their investigations on the antihemorrhagic factor and expressed the opinion that the hemorrhagic condition and the gizzard lesions were not manifestations of the same deficiency since the former often occurred in the absence of the latter and vice versa. We began our studies of the gizzard erosions because it was found that this condition was a

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complicating factor in our experiments on vitamin B<sub>4</sub> deficiency in chicks (Kline et al., '36). The results of these studies were presented at the meetings of the American Society of Biological Chemists in March, 1936 (Bird et al., '36). It was stated that the anti-gizzard-erosion factor occurred abundantly in pork lung, liver and kidney, from which sources it was not extractable by ether, ethyl alcohol or water. The factor was brought into solution by treating fresh pork lung with 0.3% NaOH and upon precipitation of the proteins by acidification to pH 4.5 was found in the protein precipitate. Oats and wheat were listed as intermediate sources of the factor and alfalfa, wheat seedlings, yellow corn, corn oil, wheat germ oil, soy bean oil, and peanuts as poor sources. The factor as it occurred in grains was found to be quite labile both to dry heat at 120°C. and to autoclaving, but was somewhat more stable in lung tissue. The insolubility of the gizzard factor in ether was presented as proof of its non-identity with the antihemorrhagic factor.

Subsequently Almquist and Stokstad ('36) reported that the gizzard lesions were prevented by feeding high levels of a hexane extract of dried kale or dried alfalfa, and that upon saponification the activity was found in the saponifiable fraction. Since the antihemorrhagic factor remains in the unsaponifiable fraction, the conclusion is drawn that the two factors are distinct. Since these results are at variance with ours, in that we found alfalfa to be a poor source of the gizzard factor and the gizzard factor to be insoluble in fat solvents, it seemed advisable to publish the details of our experiments.

#### EXPERIMENTAL

Day-old chicks were obtained from the department of poultry husbandry for these experiments. Each group was placed in a 14 inch by 16 inch by 14 inch cage individually heated and equipped with a raised wire screen bottom. The ration was supplied in round, covered metal feeders in which the feed was available to the chicks through holes in the cover. For

the most part groups of four chicks were started on the various rations and after 3 weeks each group was reduced to three chicks, and the gizzards of the chicks killed were examined. This practice was followed in order to allow for deaths during the first few days of the experiment and to prevent overcrowding during the last part of the experiment, since the cages used were not large enough for four chicks after the third or fourth weeks. The remaining chicks were killed at 5 weeks of age and the gizzards examined. The gizzards were graded according to the severity of the lesions as none or slight, marked and severe; and the gizzard factor potency of any dietary supplement was judged by its ability to produce gizzards with slight or no lesions as compared with gizzards having marked or severe lesions from the chicks on the basal ration. There are obvious defects in such a method, but it appeared to be the best available. It was not possible to use complete prevention of gizzard erosion as a criterion since we have only rarely found chicks under 6 weeks of age with gizzards entirely free of lesions, even on the best rations. The grading of the gizzards was done by the same person throughout these experiments, thus ruling out the factor of personal variation. The length of the experiments was set at 5 weeks because experiments in which chicks were killed at weekly intervals showed that the lesions continued to increase in severity up to 5 or 6 weeks of age. The greatest increase in severity was during the third week, corresponding to a marked falling off in food consumption.

As stated above, our earlier experiments on the gizzard factor were directed toward preventing gizzard erosion in chicks used in vitamin B<sub>4</sub> experiments. During the process of modification of the vitamin B<sub>4</sub> basal ration, a considerable amount of data on the gizzard factor was accumulated, and some of this material is presented here. These experiments were conducted in essentially the same manner as described above except that the chicks were killed at 6 weeks of age rather than at 5 weeks.

The basal rations used in these experiments are given in table 1. The grain rations 351 and 351E were not sufficiently

TABLE 1

*Composition of rations*

INGREDIENTS	RATION 351	RATION 351E	RATION 441	RATION 449	RATION 450H	RATION 454	RATION 456
Yellow corn	58						
Yellow corn, autoclaved 15 pounds 5 hours		55					
Standard wheat autoclaved 15 pounds 5 hours	25						
Standard wheat middlings, autoclaved 15 pounds 5 hours		25					
Dextrin			53.5	70			
Dextrin, heated 120°C. 24 hours							
Crude casein	12	12	24		69	61	60
Crude casein, autoclaved 15 pounds 5 hours							
Crude casein, heated 120°C. 24 hours					18	18	18
Reprecipitated casein				18			
Baker's yeast	1		8				3
Baker's yeast, ether extracted		4					
Brewer's yeast				1	2	2	2
Liver residue, autoclaved 15 pounds 10 hours			10	2			
Liver residue, autoclaved 15 pounds 10 hours heated 120°C. 24 hours					2	2	
Liver extract				2	2	2	
NaCl	1	1					
CaCO <sub>3</sub>	1	1					
Ca <sub>3</sub> (PO <sub>4</sub> ) <sub>2</sub>	1	1					
Salt mixture 1				5	5	5	5
Salt mixture 40			2.5				
Peanuts							
Cod liver oil	1	1	2	2	2	10	10
						2	2

low in the gizzard factor to be satisfactory basal rations, but 351 was used with some success in experiments on the heat stability of the factor, the heat treatment being applied to the corn-middlings-casein part of the ration. Rations 441, 449 and 450H were used as basal rations in vitamin B<sub>4</sub> experiments. With the exception of 441 which contains 24% of crude casein they are relatively low in the gizzard factor. Rations 454 and 456 have proved most satisfactory for basal rations in this work. It was found necessary to include 10% of peanuts, as indicated in the table, in order to supply sufficient vitamin B<sub>4</sub>. The brewer's yeast, obtained from Premier-Pabst Co. was added primarily as a source of vitamin B(B<sub>1</sub>). The liver extract in ration 454 was added primarily as a source of vitamin G(B<sub>2</sub>).<sup>2</sup> It had the disadvantages of being very hygroscopic and also rather expensive. In ration 456 the vitamin G requirement was met by including 3% of baker's yeast (Anheuser-Busch, strain C), making the total yeast content 5%.

Representative data on the distribution, stability and solubility of the gizzard factor are presented in tables 2, 3 and 4 respectively. The number of basal rations used made it difficult to summarize briefly the results, and much material has been omitted from these tables for the sake of brevity. Practically all the results presented have been confirmed by experiments with other basal rations.

Table 2 clearly shows the effectiveness of pork lung, liver and kidney as sources of the gizzard factor. The materials designated as 'vacuum dried' were obtained from Dr. David Klein of the Wilson Laboratories, and the other meat products were obtained fresh from a local packing plant and dried at 50°C. Among the grains the superiority of oats is evident, although wheat bran and wheat middlings were also good sources of the anti-gizzard erosion factor and somewhat superior to wheat itself. Dried leafy plant material, represented in the table by alfalfa and wheat seedlings, was not

<sup>2</sup> We are indebted to Dr. Harold Levine, Premier Pabst Corporation, Milwaukee, Wisconsin, for supplying the brewer's yeast and to Dr. David Klein, Wilson Laboratories, for supplying the liver extract.

BASAL RATION	SUPPLEMENT	AVERAGE WEIGHT 8 WEEKS	AVERAGE WEIGHT 5 WEEKS	AGE KILLED	CHICKS WITH GIZZARD LESIONS		
					Slight or none	Marked	Severe
441	None	gm. 86	gm. 148	weeks 6	1	5	
441	10% dried wheat seedlings	115	218	6		2	
449	32% wheat	62	102	6			2
449	32% wheat + 5% vacuum dried lung	132	202	6	2		
449	32% yellow corn	92	166	6			2
449	32% yellow corn + 5% vacuum dried lung	115	202	6	2		
450H	None	68	...	4		1	2
450H	12% alfalfa	68	...	4	1	1	1
450H	5% H <sub>2</sub> O extracted lung	77	...	4	2	1	
450H	5% H <sub>2</sub> O extracted lung + 12% alfalfa	100	...	4	1	2	
454	None	72	130	5	1	4	4
454	15% pork lung	126	265	5	6		
454	15% vacuum dried stomach	97	212	5	2	1	
454	32% whole oats	114	237	5	6		
454	32% hulled oats	97	222	5	2	1	
454	32% rolled oats	125	252	5	2	1	
454	32% wheat	87	200	5	2	1	
454	32% Wisconsin yellow corn	90	188	5		2	1
454	32% Texas yellow corn	88	188	5	1		2
456	None	71	137	5		7	3
456	32% whole oats	129	247	5	9	1	
456	5% gastric mucin granules	90	180	5	2		
456	32% wheat bran	110	222	5	3		
456	32% standard wheat middlings	110	190	5	2	1	
351E	None	131	213	5	2	2	3
351E	15% pork liver	156	304	5	7		1
351E	15% pork kidney	143	307	5	6		
351E	15% pork heart	159	266	5	4	3	1

effective. Other materials, not mentioned in the table, which gave negative results were dried blue grass, peanuts, soy bean oil, and wheat germ oil.

That the factor is destroyed to a considerable extent either by dry heat or by autoclaving is indicated in table 3. The more thorough destruction in grains, than in lung may be due to a difference in the amounts originally present. The factor as it occurs in lung is more labile at a pH of 7.5 than at natural pH.

Table 4 on the solubility of the gizzard factor indicates the complete failure of attempts to extract it with ether, alcohol, or water. The water extract of lung was prepared by adding cold water to the lung tissue, heating to the boiling point and filtering hot. The first success in bringing the factor into solution was achieved by treating fresh pork lung with 0.3% NaOH. The gizzard factor potency appeared in the precipitate resulting when the NaOH solution was acidified to pH 4.5.

Fresh pork lung was also fractionated according to the method of Siegfried ('02) for the preparation of reticulin, it having been noted that the three best sources of the gizzard factor, lung, liver and kidney are also the organs containing the largest amount of reticular connective tissue. The lung tissue was digested with trypsin, the residue extracted with ether, the ether residue boiled 20 minutes with 0.05% HCl, filtered, and the residue washed once with warm water. The filtrate was designated the gelatin fraction and the residue the reticulin fraction. The activity, as shown in the table, appeared in the latter fraction.

An attempt to use the NaOH treatment and subsequent acid precipitation with oats instead of with lung was unsuccessful, the activity being distributed among the three fractions.

TABLE 3  
*Stability of the gizzard factor*

BASAL RATION	MODIFICATION	AVERAGE WEIGHT 3 WEEKS	AVERAGE WEIGHT 5 WEEKS	AGE KILLED	CHICKS WITH GIZZARD LESIONS		
					Slight or none	Marked	Severe
351	None	gm. 129	gm. 252	<i>weeks</i> 5-6	13	1	
351	Heated 60°C. 24 hours	141	251	6	5		
351	Heated 80°C. 24 hours	114	219	5-6	4	9	1
351	Heated 100°C. 24 hours	97	206	5	3	2	4
351	Heated 120°C. 24 hours	93	148	6	1	2	1
441	None	90	158	6		3	
441	24% yellow corn	103	207	6	3		
441	32% yellow corn	93	195	6	2		1
441	24% yellow corn, heated 120°C. 24 hours	90	155	6		3	
441	32% yellow corn, heated 120°C. 24 hours	90	198	6		3	
441	10% vacuum dried lung	109	164	6	3		
441	15% vacuum dried lung, heated 90°C. 24 hours	165	280	6	2		
441	15% vacuum dried lung, heated 120°C. 24 hours	142	250	6	1	2	
454	None	60	123	5		1	2
454	15% vacuum dried lung, heated 120°C. 24 hours	100	217	5		1	1
454	15% vacuum dried lung, autoclaved 15 pounds 5 hours	72	122	5	1	2	



TABLE 4  
*Solubility of the gizzard factor*

BASAL RATION	SUPPLEMENT	AVERAGE WEIGHT, 3 WEEKS	AVERAGE WEIGHT, 5 WEEKS	AGE KILLED	CHICKS WITH GIZZARD LESIONS		
					Slight or none	Marked	Severe
		<i>gm.</i>	<i>gm.</i>	<i>weeks</i>			
351E	None	131	213	5	2	2	3
351E	Ether extract of 15% pork liver	114	211	5	1	4	2
351E	Ether extract of 15% pork kidney	104	200	5	2	1	4
351E	Ether extract of 15% pork heart	113	216	5	5	2	2
351E	Ether extract of 10% dried alfalfa	104	181	5	1	3	4
441	Ether extract of 15% vacuum dried lung	100	183	6		3	
441	Ether residue of 15% vacuum dried lung	147	270	6	2		
441	95% alcohol extract of 15% vacuum dried lung	108	201	6		3	
441	95% alcohol residue of 15% vacuum dried lung	137	277	6	3		
450H	None	68	...	4		1	2
450H	5% H <sub>2</sub> O extracted residue of vacuum dried lung	77	...	4	2	1	
454	None	74	139	5	1	4	4
454	15% pork lung	126	265	5	6		
454	NaOH residue of 15% pork lung	90	177	5		2	1
454	Acid ppt. of NaOH extract of 15% pork lung	79	142	5	6		
454	Acid soluble fraction of NaOH extract of 15% pork lung	60	95	5		1	1
454	Trypsin digest of 15% pork lung	107	190	5		3	
454	Ether extract of trypsin residue of 15% pork lung	73	140	5		2	1
454	Gelatin fraction of 15% pork lung	63	118	5	1	1	1
454	Reticulin fraction of 15% pork lung	85	162	5	3		
456	None	61	112	5		2	1
456	32% whole oats	115	257	5	2		
456	NaOH residue of 32% whole oats	77	143	5	2	1	
456	Acid ppt. of NaOH extract of 32% whole oats	89	162	5	2	1	
456	Acid soluble fraction of NaOH extract of 32% whole oats	84	192	5	3		

## DISCUSSION

The divergence in the results obtained with alfalfa by Almquist and Stokstad and by us is difficult to explain, even on a quantitative basis, although there was a considerable difference in the amounts fed. Almquist and Stokstad found that their chicks were protected by the hexane extract equivalent to 25% alfalfa. Upon adding 12% of alfalfa to ration 450H we found only very doubtful improvement in the gizzard lining, and the addition of the same level of alfalfa to ration 450H plus 5% water extracted lung increased rather than decreased the severity of the gizzard lesions. Likewise the addition to ration 351E of the ether extract equivalent to 10% alfalfa seemed to increase the severity of the lesions. The work with leafy plant tissue was, in general, very unpromising since grass and wheat seedlings also tended to increase the severity of the gizzard lesions.

Pork lung has proved thus far to be the most satisfactory source of the factor, and here as in other sources the factor was entirely unextractable by ether. The finding of the gizzard factor potency in the acid precipitable fraction of the NaOH extract of lung, indicates a relation to the proteins, but whether the factor is chemically combined in the protein molecule or merely adsorbed, it is of course impossible to say. The results of the preparation of reticulin from lung tissue show the association to be with the more resistant protein, not hydrolysed by trypsin. The possibility of relationship between the protein of the gizzard lining and the reticulin of lung, liver and kidney leads to interesting speculations; however, the results obtained upon the fractionation of oats show that the factor is not necessarily a part of a protein molecule.

The superiority of oats over the other whole grains studied as a source of the gizzard factor is likely to be of considerable practical importance. The possibility of improving, under certain conditions, the growth of young chicks by the addition of oats to the ration has been recognized for some time. Wilcke ('36) has recently reported data of this nature. However, so far as is known, no satisfactory explanation of these results

has until now been supplied. Gizzard erosion has been observed quite extensively under practical conditions, which is not surprising in view of the general use of corn, a poor source of the gizzard factor, in poultry rations.

At an early stage in this work, the heat lability of the factor as it occurs in grains suggested the possibility of its identity with vitamin B<sub>3</sub>. The later discovery of the greater stability of the factor as it occurs in lung seemed to argue against this possibility, but this greater stability may have been due merely to a greater concentration originally present. Attempts to concentrate the factor from lung by a method similar to that used by O'Brien ('34) for the concentration of B<sub>3</sub> from wheat germ were unsuccessful, but this may have been due to obvious differences in the source material. A pigeon experiment gave inconclusive results although pigeons fed on polished rice supposedly deficient in vitamin B<sub>3</sub> did show slight gizzard lesions. Disagreements in the literature as to the properties of vitamin B<sub>3</sub> make it difficult to establish definitely the identity or non-identity of the two factors. It seems possible that the condition of poor growth in chicks described by Eddy, Gurin and Keresztesy ('30) and supposedly a vitamin B<sub>3</sub> deficiency may have been a gizzard factor deficiency.

#### CONCLUSIONS

It may be concluded that the anti-gizzard-erosion factor is distinct from the antihemorrhagic factor required by chicks; that it occurs abundantly in pork lung, liver and kidney, and in a descending order of abundance in oats, wheat and corn; that wheat bran and wheat middlings compare favorably with oats as sources of the factor; that it exhibits some variation in heat stability depending on the source; and that it is insoluble in ether and in ethyl alcohol but follows the alkali soluble acid precipitable proteins in the fractionation of lung tissue.

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# THE USE OF A 10-DAY PERIOD FOR THE ASSAY OF VITAMIN B BY RAT GROWTH TECHNIC <sup>1</sup>

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SIX FIGURES

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The need for a short time method of assaying vitamin B<sup>2</sup> developed during a study of the vitamin B retentions of children. Because the quantities of urine and feces available from the children during a metabolism period were definitely limited, it was impossible to employ the 8-week, or even 4-week, method of assay recommended by Chase and Sherman ('31). In order to determine whether growth for 10 days is a valid criterion of the amount of vitamin B in the material being tested, a study has been made of the factors which might cause variability in results. While several of these factors have previously been reported by investigators of vitamin B requirements, they have not all been studied and compared under the same technic, nor evaluated in relation to the growth of the animal.

## FACTORS AFFECTING THE LENGTH OF THE DEPLETION PERIOD

*Ration ingredients.* Rats, whose weights were within the range of 40 to 45 gm., were weaned at 21 days of age and placed in individual cages with raised screen bottoms. Groups consisting of three males and three females (no two rats

<sup>1</sup> The preliminary work for this study was done at the Iowa Child Welfare Research Station, Iowa City, Iowa.

<sup>2</sup> The term vitamin B has been used for the fraction of the vitamin B-complex sometimes designated vitamin B<sub>1</sub>.

from the same litter) were given either the following ration A or a variation of it.

<i>Ration A</i>			
	<i>gm.</i>		<i>gm.</i>
Casein <sup>3</sup>	18.0	Cod liver oil	2.0
Dextrin <sup>4</sup>	60.5	Butterfat (filtered)	5.0
Agar	2.0	Crisco	8.0
Salts <sup>5</sup>	4.5		
Autoclaved yeast <sup>6</sup>		0.5 gm. per day	

Ration A was chosen because of its similarity to the ration recommended by Chase and Sherman. The variations were partly chosen because of the report of Bender, Flanigan and Supplee ('34) in regard to the use of autoclaved whey, and the report of Guerrant, Dutcher and Tomey ('35) in regard to the source of carbohydrate. The changes in the ration were a comparison of autoclaved whey<sup>7</sup> with autoclaved yeast, an increase of the supplement used to supply vitamin G, substitution of sucrose for dextrin, and omission of agar and fat. The rats were weighed weekly and observed daily for symptoms.

The outstanding result was the variation in the length of time before polyneuritis developed and the animals died. Figure 1 records this variation by giving the number of days before death for the individual rats.

<sup>3</sup> Vitamin free casein was obtained from the Casein Mfg. Co., New York.

<sup>4</sup> Tapioca was autoclaved for 4 hours at 16 pounds pressure and ground.

<sup>5</sup> Salts consisted of McCollum no. 185 plus 17.7% calcium carbonate and 2.86% ferric citrate.

<sup>6</sup> Yeast was prepared by mixing 190 gm. Mead Johnson Yeast with 50 cc. N NaOH and 200 cc. water. After standing overnight in a refrigerator the yeast mixture was autoclaved for 5 hours at 16 pounds pressure, dried and ground. This process was then repeated with the single change of only 2 hours autoclaving.

<sup>7</sup> Dried whey (Peebles Lacto Milk) was furnished through the courtesy of the Western Condensing Co., San Francisco. The whey was mixed with an equal weight of distilled water and autoclaved, in shallow pans, for 2 hours, at 16 to 18 pounds pressure.

## Variations in Depletion due to Ration Changes

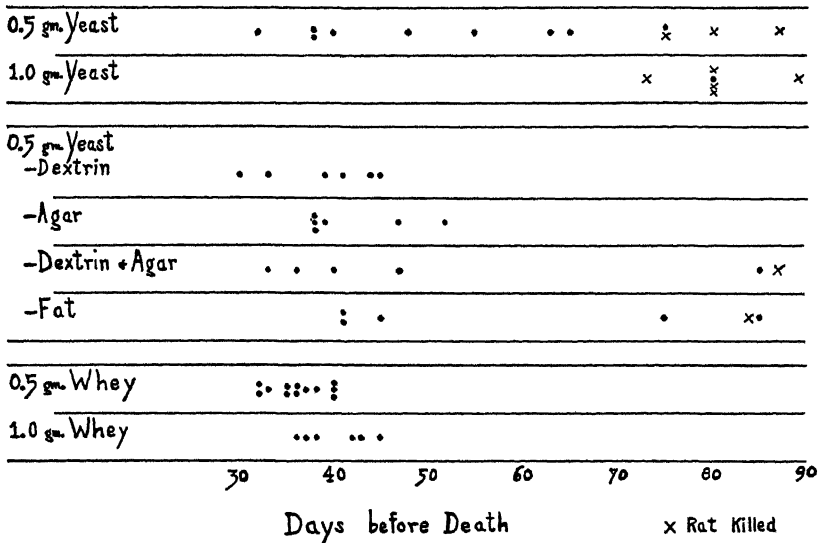


Figure 1

The results may be summarized as follows:

RATION		NUMBER OF RATS		NUMBER OF DAYS BEFORE DEATH	
Supplement	Change	Living	Dead	Range	Average
0.5 yeast	.....	3	9	32-75	51
1.0 yeast	.....	5	1	80-	80
0.5 yeast	No dextrin	0	6	30-45	39
0.5 yeast	No agar	0	6	38-52	42
0.5 yeast	No dextrin or agar	1	5	33-85	48
0.5 yeast	No fat	1	5	41-85	57
0.5 whey	.....	0	12	32-40	36
1.0 whey	.....	0	6	36-45	40

It will be noted that a change in the daily supplement from autoclaved yeast to autoclaved whey produced more uniformity in results than did the substitution of sucrose for dextrin in the ration or the omission of agar. Furthermore, an increase of autoclaved whey from 0.5 gm. daily to 1.0 gm. did not greatly prolong the life of the animal as did a similar increase

in the daily dose of autoclaved yeast. The effect of the additional yeast in preventing polyneuritis and death of the rats is not thought to be due to a residual content of vitamin B since the yeast was double autoclaved under rigid alkaline conditions.

On the basis of the above results, the following ration B was selected for further study:

<i>Ration B</i>	
	<i>gm.</i>
Purified casein	20.0
Autoclaved whey	15.0
Cane sugar	38.0
Salts	5.0
Cod liver oil	3.0
Crisco	19.0

*Initial weight of rat.* To test the influence of the size of the rat upon the length of the depletion period, groups of rats were weaned at 21 days and placed on stock diet until they had attained weights of about 50, 60, 80, 100 or 120 gm., at which time they were transferred to the vitamin B free ration. The average time before death for each group was as follows:

<i>Average initial weight</i>	<i>Number of rats</i>	<i>Average days before death</i>
43	11	32.0
50	7	37.3
61	18	34.3
81	5	39.2
102	6	35.0
121	6	39.3

While there was not a great deal of difference in results for these different weight groups, a slightly more uniform response was observed for the group averaging about 60 gm. than for smaller or larger rats.

#### FACTORS AFFECTING THE GROWTH RESPONSE OF THE RATS

*Ration ingredients.* Following a depletion period of about 3 weeks, doses of stabilized wheat germ,<sup>s</sup> have been fed for

<sup>s</sup> A wheat germ product, stabilized through removal of fat and some moisture, containing approximately 15 Sherman units of vitamin B per gram, has been supplied by the VioBin Corporation, Chicago, Illinois.



8 weeks to two series of rats. Series I consisted of seven groups (six rats to a group) which received ration A. In addition, the animals of these groups were fed daily (except Sunday) 0, 0.0375, 0.05, 0.1, 0.2, 0.5, or 1.0 gm. of the stabilized wheat germ. Series II consisted of six groups of rats which received ration B and wheat germ as follows: 0, 0.1, 0.2, 0.3,

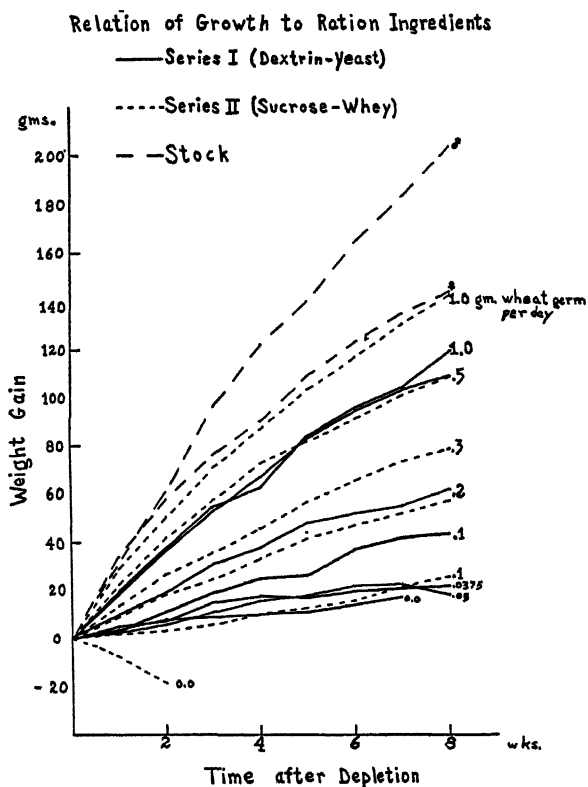


Figure 2

0.5, or 1.0 gm. per day. The growth curves for the 8 weeks after depletion for both series are presented in figure 2. The rats of series I, which had received dextrin and autoclaved yeast, grew better at the lower levels of wheat germ and more poorly at the higher levels than did the rats of series II, which had been fed sucrose and autoclaved whey. Apparently

the yeast-dextrin combination enabled the rats to grow to a certain extent regardless of insufficient quantities of vitamin B in their daily supplements, but was not adequate to support growth when larger amounts of vitamin B were administered.

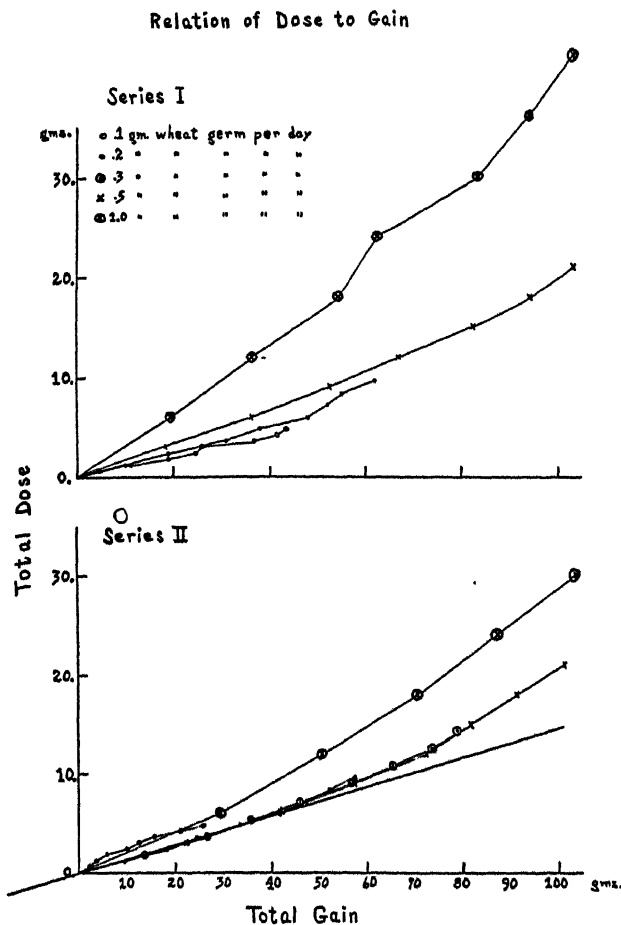


Figure 3

*Size of dose.* In an attempt to analyze further the growth response of these two series of rats, the total accumulative amount of dose per rat, at the end of each week, for each group, has been plotted in figure 3 against the average accumulative gain for the corresponding period. As has been

previously commented, poor growth resulted in series I in the group receiving 1.0 gm. of wheat germ per day. A study of figure 3 shows that this poor growth was the result of a progressive increase in the amount of dose required for a gram of gain as the ingestion of wheat germ was increased from 0.1 to 0.2, 0.5 and 1.0 gm. per day. The 0.05 and 0.0375 gm. groups were not plotted since their response was similar to the negative controls and was obviously influenced by the dextrin and autoclaved yeast of the ration.

In series II very consistent results were obtained for the 0.2, 0.3 and 0.5 gm. per day groups. While there was a slight upward trend in the amount of dose required for gain toward the end of each of these three levels of dose, a very close direct relation of gain to dose was observed. The lower portions of each curve coincided in a straight line showing that 1 gm. of gain had required 0.145 gm. of the wheat germ. Both the 1.0 and the 0.1 gm. groups required slightly more dose for a unit gain. Since the growth curves of the 1.0 gm. group are nearly comparable to those for stock rats (fig. 2), it is probable that this lessened gain per unit of dose for the 1.0 gm. group was due to a lack of further growth capacity. Additional tests, however, of the basal ration are being conducted to ascertain more definitely whether it may have any limitations for growth. The poor response of the 0.1 gm. per day group may be due to the low level of the dose. In other experiments with vitamin B assays it has been observed that rats receiving border line doses have a tendency to become too greatly depleted. Such rats must restock their body stores to a certain extent before growth is resumed. While the rats of this 0.1 gm. per day group were not depleted to the point of polyneuritic symptoms such as incoordination of movements or paralysis, in all probability they were not receiving at first sufficient vitamin B for regular growth. Gradually, however, their condition improved until at 8 weeks their response approached that for the next two groups. The fact that this inferior response for the 0.1 gm. per day group was not particularly noticeable when the growth curves were plotted

in figure 2, but became apparent only when the amount of gain was analyzed in relation to the dose, would seem an important consideration in evaluating the results of a vitamin B assay.

The relation of gain to dose has therefore been subjected to further analysis. In figure 4 the logarithms of the total

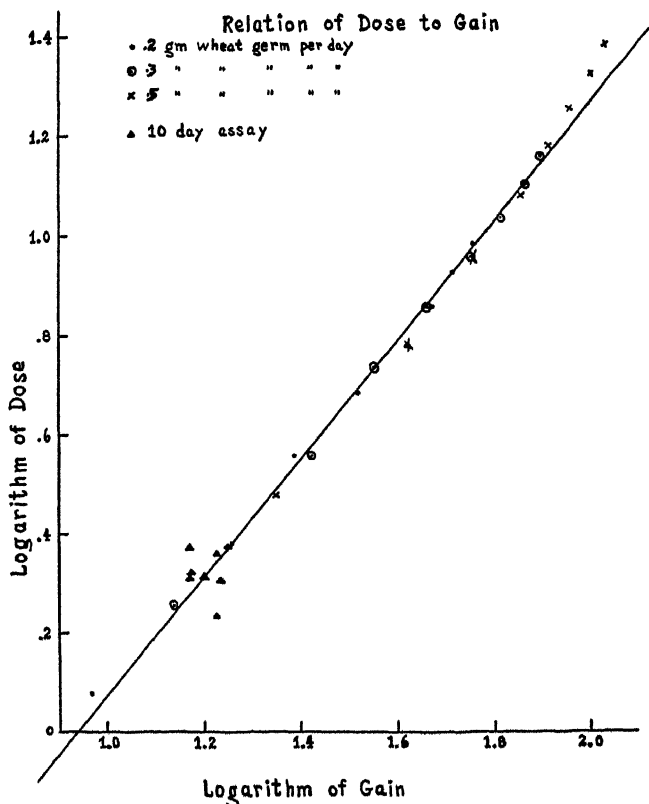


Figure 4

amounts of dose at the end of each week have been plotted against the logarithms of the total gains. This method of analysis brought out a greater degree of direct dependence of gain upon dose than was apparent in figure 3. Nearly all of the slight upward curves observed in figure 3 were eliminated and a longer straight line resulted when the loga-

arithms of dose and gain were used for 0.2, 0.3 and 0.5 gm. groups. Equations developed from the straight line of figure 4 gave the following results:

$$\text{Dose} = 0.0773 (\text{gain})^{1.186}$$

$$\text{Gain} = 1.186 \sqrt[1.186]{\text{dose} + 12.95}$$

*Weight of rat.* Since the total gain at any time appears to be directly related to the accumulative effect of the total dose at that time, the amounts of dose required for 1 gm. of gain have been calculated at the end of each week for the rats of series II. When these dose per gram of gain figures were

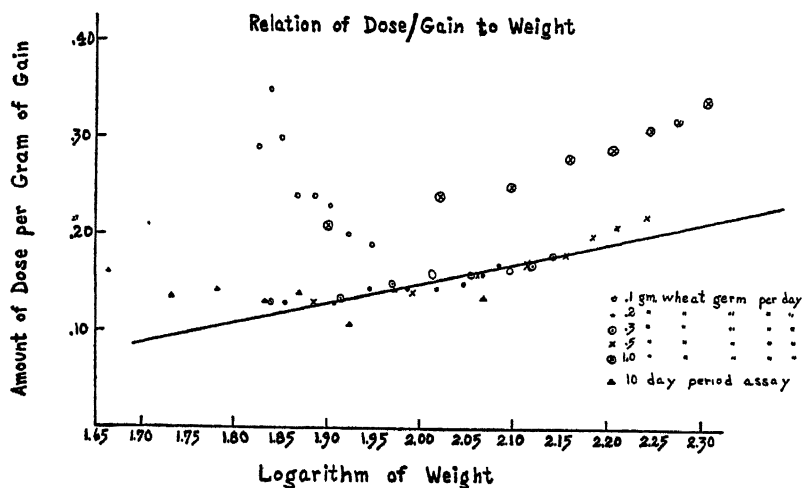


Figure 5

plotted against the average weights of the rats for each week, a series of curved lines resulted. Cowgill ('34) has stated that the vitamin B requirement is related to the  $5/3$  power of the weight. The dose per gram of gain figures have therefore been plotted in figure 5 against the logarithms of the average weights for each week. This treatment straightened somewhat the curves obtained by use of unaltered weight figures, but did not indicate as direct a relation between dose/gain and weight as was observed between dose and gain. Thus the dependence of gain upon the size of the dose would

appear to be the fundamental consideration, although the constants included in the equations developed from figure 4 may be partially influenced by the increasing weight of the animal. Further studies of the relationship between size of dose, rate of gain, and weight of rat are now in progress, that sufficient data may be obtained for more accurate calculation of equations to determine vitamin B requirement.

The inferior response of the 0.1 gm. group must again be mentioned in view of the results obtained from figure 5. The amount of dose required for 1 gm. of gain was definitely more for this group than for either the 0.2, 0.3 or 0.5 gm. groups. It would seem that the 0.1 gm. level was too low and that the rat was unable to utilize the vitamin B of the wheat germ for growth because of other possible physiologic needs.

*Length of the dose administration period.* From the relation of dose to gain as plotted in figures 3 and 4 it would appear that the amount of gain is proportional to the amount of dose regardless of the length of the dose administration period. Valid estimates of the vitamin B content of a material may be obtained from short assay periods by merely calculating the amount of dose per unit of gain if rats of the same size have been used whose rates of gain were controlled within the limits of the 0.2 to 0.5 gm. groups, to eliminate the slight influence of weight.

#### TEN-DAY ASSAY PERIODS

The use of a 10-day dose administration period has been studied in a third series of rats. Eight groups of different sized rats, ranging in weight from 40 to 120 gm., were depleted of their vitamin B stores by means of ration B. When an animal was losing weight regularly, doses of the stabilized wheat germ were administered for a period of 10 days. The amount of dose to be given was determined each day from the gain of the rat for the previous day in order to keep the rate of gain within the limits of 1 to 2 gm. per day. This rate of gain was comparable to that of the 0.2 to 0.5 gm. groups of series II and was therefore directly proportional to the amount

of vitamin B in the dose. At the end of 10 days doses were discontinued, but the animal was weighed daily until it was again losing weight. If the rate of gain had been carefully controlled within the limits of 1 to 2 gm. per day, the rat usually started to lose weight the day after dose was stopped. Total dose was then divided by total gain to obtain a dose per unit gain figure.

The average amounts of dose required for a gram of gain for the different weight groups of series III were as follows:

WEIGHT GROUP	NUMBER OF RATS	TOTAL GAIN	TOTAL DOSE	AMOUNT OF DOSE FOR 1 GM. GAIN
42.0- 48.0	6	14.8	2.36	0.162 <sup>1</sup>
51.0- 59.0	12	15.8	2.08	0.137
60.0- 64.5	17	14.8	2.05	0.144
65.5- 69.0	11	17.1	2.02	0.131
70.0- 77.0	5	14.8	2.07	0.140
80.0- 87.0	6	16.9	1.71	0.107
92.0- 99.0	6	16.9	2.33	0.142
100.0-135.0	4	18.0	2.40	0.135

<sup>1</sup> The animals of this smallest weight group seemed to become depleted more severely in relation to their losses of weight than rats in other groups.

The similarity in results for these averages for different groups would seem to indicate that valid results may be obtained from assay periods as short as 10 days when the rate of gain of the rat is controlled.

When the logarithms of the total dose and total gain for the 10-day assay groups were included in figure 4, they coincided with the straight line obtained from the figures for the 0.2, 0.3 and 0.5 gm. groups of series II, giving further proof that accumulative gain is dependent upon accumulative dose and is not related to the duration of the dose administration period. A relation between the weight of the rat and the amount of dose required for a unit gain was not apparent over the range studied since similar results were obtained for all weight groups. More irregularity, however, was encountered in the daily gains of the individual rats from the larger and smaller weight groups than for rats started at an initial weight of about 60 gm. Further experiments, particularly at higher

levels of weight, are now in progress to test more exactly the relations between animal weight and vitamin B requirement.

#### CONTROL EXPERIMENTS WITH CRYSTALLINE VITAMIN B

A series of twenty-one rats (initial weight 60 gm.) were given ration B until they were depleted of vitamin B stores. When the rats showed consistent losses of weight on consecutive days, doses of crystalline vitamin B<sup>9</sup> were given daily. The average response of the rats (fig. 6) was comparable to

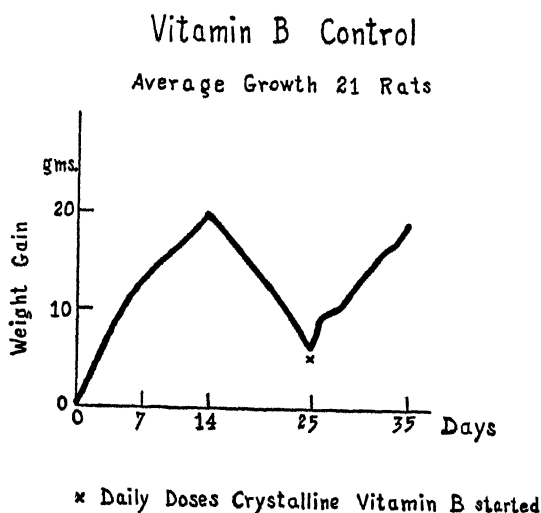


Figure 6

the growth obtained by Waterman and Ammerman ('35) at similar levels of intake since an average of 4  $\gamma$  produced 1 gm. of gain.

#### SELECTION OF A UNIT FOR SHORT PERIOD ASSAYS

The 0.1 gm. group of series II (fig. 3) had a growth rate comparable to the 3 gm. per week recommended by Chase and Sherman as desirable for estimating 1 unit of vitamin B. The inferior response of this group, however, when growth

<sup>9</sup> Crystalline vitamin B was furnished through the courtesy of Merck & Co., Rahway, New Jersey.



was analyzed in relation to the amount of dose required for a gram of gain (as illustrated in figs. 3 and 5), may indicate that this growth rate is too near the borderline of requirement to be dependable as a unit. The consistent agreement between amount of dose and amount of gain for the 0.2, 0.3, and 0.5 gm. groups and for the 10-day assay groups when the rate of gain was kept within the limits of 1 to 2 gm. per day, would appear to justify the conclusion that a more satisfactory unit would be the amount of vitamin B producing 1 gm. of gain. This new unit is comparable in magnitude to the minimum curative dose described by Ammerman and Waterman ('35), and is approximately equal to two Chase and Sherman units.

#### SUMMARY

A short period technic for vitamin B assays has been developed through a study of the factors producing variability in results.

The amount of growth of the rat during 10 days was found to be a valid criterion of the quantity of vitamin B in the dose material if the following precautions had been observed.

1. The basal ration contained autoclaved whey in preference to autoclaved yeast, and sucrose instead of dextrin.
2. The rats weighed about 60 gm. when started on depletion.
3. The amount of dose was controlled each day to keep the rate of gain of the rats between 1 and 2 gm. per day.

The quantity of vitamin B causing 1 gm. of gain, when the rate of gain has been limited to 1 to 2 gm. per day, is suggested as a unit since it is comparable to the minimum curative dose defined by Ammerman and Waterman.

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# A QUANTITATIVE STUDY OF THE UTILIZATION AND RETENTION OF VITAMIN B BY YOUNG CHILDREN <sup>1, 2</sup>

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TWO FIGURES

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Recent studies have indicated that an increase in vitamin B ingestion is frequently beneficial to infants and children. Gaynor and Dennett ('34) fed 100 normal infants a dried milk reinforced with a specially prepared water extract of rice polish as a source of vitamin B for an average period of 5 months. Fifty additional infants were fed an unfortified dried milk. In reference to the effect of the vitamin B extract, Gaynor and Dennett state:

The increase in weight was influenced favorably; metabolic efficiency was increased; anorexia and gastrointestinal disturbances were lacking; pallor was less marked; and nutrition was improved and a greater resistance to infection exhibited. They were as a group mentally more alert and less irritable, sleeping better and possessing practically none of the common complaints of infancy.

Morgan and Barry ('30) have reported favorable results when the vitamin B intake of under-weight children was increased. They obtained weight gains from 150 to 170% above

<sup>1</sup> The data of this study are taken from a dissertation submitted in partial fulfillment of the requirements for the degree of doctor of philosophy in nutrition, Child Welfare Research Station, State University of Iowa.

<sup>2</sup> The term vitamin B has been used for the fraction of the vitamin B complex which is sometimes designated vitamin B<sub>1</sub>.

the expected gain when they fed two wheat germ rolls at the noon meal, while the control group which received white flour rolls gained only 50 to 71% of the expected gain during the same period.

Summerfeldt ('32) has likewise successfully applied vitamin B therapy to underweight children. Gains of 4.54 and 5.27 pounds in 10 weeks were produced by administering each week 4.2 ounces of wheat germ and 0.28 ounce of yeast, incorporated in a special breakfast cereal. The control groups, receiving ordinary cereals, gained only 1.25 and 1.27 pounds. That the gain with the special cereal was in part due directly to its vitamin B content has been shown by Ross and Summerfeldt in a more recent study ('35) where a third group received a vitamin B concentrate in addition to the ordinary cereal. The gain for this group was 5.5 pounds in 6 months as compared to gains of 3.6 pounds for the group receiving ordinary cereals and 7.8 pounds for the group receiving the special cereal.

If the prevalence of anorexia may be considered as an indication of inadequacy in regard to vitamin B, the following reports by Bartlett ('28) and Schlutz ('25) have particular significance in a study of children's requirements. After examination of 1471 consecutive records for children brought to the outpatient clinic, Bartlett concluded that anorexia far exceeded all other presenting symptoms in its incidence. Schlutz, in analyzing the role of the diet in the treatment of disorders of older infants and children, has said, "A complaint for which the child is brought to the physician more often than any other is persistent anorexia with cessation of gain in weight. . . . A problem is to provide abundant vitamin supply, particularly the water soluble vitamin B."

The evidence available, therefore, appears to indicate that dietary vitamin B for children frequently may be less than the amount required for optimum health. Thus a quantitative investigation of the utilization and retention of vitamin B by young children, as influenced by different levels of ingestion, seemed of value in determining requirements more exactly.

## EXPERIMENTAL PROCEDURE

*Children*

The utilization of vitamin B has been studied through biological assay with rats of the amounts of vitamin B in the food and excreta of children receiving weighed diets. A total of twenty-three balance studies have been completed with eight children of 4 to 7 years of age. The children, who were selected from private homes and from a county juvenile home, were apparently in normal health. While being studied they were under constant supervision by nurses trained in metabolism technic.

*Experimental period.* The experimental period usually lasted 3 weeks during which time the same foods were served each day, the amount for each child being in proportion to his height and theoretical weight. To allow for adjustment to the level of vitamin B in the diet, the experimental period was divided, with one exception, into a 6-day preliminary period preceding two successive collection periods of either 4 or 5 days each. Extreme care was used to insure that the individually weighed portions of food were quantitatively consumed and the urine and feces quantitatively collected. The exception of the 6-day preliminary period was that related to the diet low in vitamin B. In this case, only a 1 day preliminary period was followed by 3 days of collections.

*Children's diets.* Three different levels of vitamin B ingestion have been tested. The diets varied chiefly in the selection of cereals and bread. For the highest and medium levels of vitamin B, whole wheat bread and cereal were contrasted with white bread and wheat endosperm cereal. For the lowest level the amount of vitamin B supplied by fruits, vegetables, and milk was decreased. The details of the various dietaries for D.G. and V.M. are given in table 1, since these children received dietary constituents comparable in kind and amount to those of the other children studied at each level of ingestion.<sup>3</sup>

<sup>3</sup>I am indebted to Miss Florence I. Scoular, research assistant, Iowa Child Welfare Research Station, for calculation of the diets and preparation of the meals.

*Preparation of materials.* All of the food for each collection period was prepared in advance for the entire period and stored in a low temperature refrigerator (8°C.), so that the servings for each day and the aliquot weighed for analysis

TABLE 1

*Foods taken by the children during representative experimental periods (amounts in grams per day)*

	11/23-25 D.G.	1/16-20 D.G.	2/25-3/1 D.G.	2/5-9 D.G.	11/14-17 D.G.	3/20-24 V.M.
Apple	80.0	139.0	85.0	35.0	139.0	159.0
Banana	50.0	126.0	41.0	31.0	126.0	126.0
Orange juice	15.0	61.5	62.7	62.6	125.0	127.2
Prunes (cooked)	....	20.1	20.1	20.1	20.1	26.4
Dates	10.0	....	16.0	....	....	....
Carrots	....	88.0	88.0	88.0	88.0	88.0
Tomatoes (cooked)	....	120.0	120.0	120.0	120.0	120.0
Onions	50.0	....	....	....	....	....
Potatoes	....	75.0	75.0	75.0	75.0	103.8
Rice (cooked)	60.0	....	....	....	....	....
Beef	60.0	60.0	60.0	60.0	60.0	36.0
Bacon	25.0	....	....	....	....	....
Egg	70.0 <sup>1</sup>	115.0	110.0	80.0	115.0	75.0
Cheese (American)	30.0	....	....	....	....	....
Milk	305.0	488.0	488.0	976.0	488.0	488.0
Butter	35.0	33.0	31.0	19.0	32.0	32.0
Bread, white	60.0	48.0	....	....	....	....
Bread, whole wheat	....	....	25.0	26.0	72.0	70.0
Muffins (whole wheat)	....	....	33.0	....	....	....
Ralston	....	....	13.2	13.8	13.8	18.0
Cream of wheat	15.0	13.7	....	....	....	....
Tapioca	13.0	....	....	....	....	....
Sugar	44.0	23.0	15.0	15.0	24.0	19.0
Salt	1.0	1.0	1.0	1.0	1.0	1.0
Cod liver oil	12.0	12.0	12.0	12.0	12.0	12.0
Vioosterol (drops)	8.0	8.0	8.0	8.0	8.0	8.0
Calories	1659.0	1615.0	1561.0	1540.0	1691.0	1650.0
Protein	53.5	57.3	62.6	64.9	59.8	51.0

<sup>1</sup> Egg white only.

might be identical in composition. Precautions, such as utilization of cooking water for vegetables, were followed to prevent, so far as possible, losses of vitamin B during the preparation of the foods. The food aliquots for the entire day to

be analyzed were mixed thoroughly with acid alcohol (95% ethyl alcohol plus 2% glacial acetic acid) and dried, first on a steam bath and finally to constant weight in an electric oven maintained at a temperature between 60° and 70°. Feces were preserved with acid alcohol and kept in a low temperature refrigerator (5°C.) until the amount for the entire period, as determined by carmine marking, was obtained. They were then pooled and dried in a manner similar to that used for food. Urine was made up to volume for each 24 hours and preserved under toluene in the refrigerator at 5°C. until pooled at the end of the collection period. It was then concentrated on a steam bath to one-twentieth of its original volume, the pH being adjusted to between 2.0 and 3.0 with hydrochloric acid.

#### BIOLOGICAL ASSAY

Young rats, weaned at about 28 days of age when their weight had reached 60 gm., were used for biological assay. These were placed in cages with raised screens ( $\frac{1}{2}$  or  $\frac{1}{3}$  inch mesh) and fed, ad libitum, the following basal depletion ration:

	<i>gm.</i>
Casein <sup>4</sup>	20.0
Autoclaved whey <sup>5</sup>	15.0
Cottonseed oil	20.0
Cane sugar	39.5
Cod liver oil	2.0
Salts—Wesson ('32)	3.5

The materials to be assayed were administered when the rats had commenced to lose weight regularly after 24 to 26 days of depletion. The dried food and feces were weighed on the analytical balance and fed directly, while the concen-

<sup>4</sup> Casein was freed from vitamins by soaking for 5 days in a 0.2% acetic acid solution changed twice daily, followed by three alternate treatments of solution in ammonium hydroxide and precipitation with acetic acid. After 48 hours of extraction in 80% alcohol, the first half hour being at 60°, the casein was dried at room temperature and ground.

<sup>5</sup> Dried whey (Peebles Lacto Milk) was obtained through the courtesy of the Western Condensing Co. of San Francisco. Four hundred grams of whey were mixed with an equal weight of distilled water, spread in a pan 9 × 15 inches, and autoclaved at its natural pH (5 to 6) for 2 hours at 18 pounds pressure.

trated urine was pipetted to tenths of a cubic centimeter and mixed with a little of the basal ration. Since the biological materials to be assayed were definitely limited in quantity, doses were administered to the rats for a period of 10 days. This method of assay was found to be valid when the size of the rat, length of the depletion period, size of the dose, and rate of gain of the rat were carefully controlled and standardized. The unit selected for comparison of results was the quantity of vitamin B causing 1 gm. of gain in the assay animals. This was calculated by dividing the total dose for 10 days by the total gain for that period. This unit was chosen because of its harmony with the minimum curative dose (Ammerman and Waterman, '35), the accuracy with which it may be calculated, and the simplicity with which it may be compared with a reference unit such as crystalline vitamin B. It is equal approximately to 2 Chase and Sherman ('31) units. The details of the assay technic have been given by Schlutz and Knott in the preceding paper.

#### DISCUSSION OF RESULTS

The detailed results of the assays for the various materials are given in table 2, at least three rats having been used to determine the vitamin B of each material. It is granted that this number of rats for an assay is less than the desired number, but the biological materials for this study were definitely limited in quantity. To offset this limitation, extreme care was used both in the technical details of the assay and in the evaluation of individual rat responses. All assays showing a too great degree of variability or a lack of harmony with other results obtained for the same level of vitamin B intake, were repeated to insure reliability of the final figures.

In assaying the amounts of vitamin B in the foods for the lower levels of intake (D.G., F.V., C.H. 11/23-25 and D.G., B.B. 1/16-20), difficulty was encountered in getting the rat to eat a large enough dose to furnish sufficient vitamin B to stimulate growth. It was necessary, therefore, to estimate from the assay data available the vitamin B content of these



foods. This procedure was considered to be more accurate than determination of the original food content by extracting the vitamin B, since such solvents as alcohol do not extract the vitamin quantitatively. The estimates were made on the basis of the amount of dose actually eaten by the rat, considering the amount which maintained weight or caused temporary growth as containing 1 unit of vitamin B. Since the weight of food containing 1 unit had to be (from the definition of a unit) greater than this largest amount of dose which was consumed without regular gain, the number of units as estimated for the daily food for D.G., F.V., and C.H. 11/23-25 and for D.G. 1/16-20 should be less than the amounts recorded in table 3.

The most significant result of the investigation was the definite trend toward higher retentions of vitamin B accompanying higher levels of intake in the children studied. These retentions have been plotted in figure 1 in relation to the level of vitamin B ingested. When the summary data for individual children, as presented in table 3, are considered it will be seen that retentions (calculated on the basis of the vitamin B of the food minus that of the urine), for the three children receiving different levels of vitamin B, increased with intake from 1 to 106 units for C.H., from 5 to 153 units for D.G., and from — 18 to 210 units for F.V. The total averages for each level of ingestion (table 4) also indicate that in spite of increasingly larger amounts of vitamin B in the urine as ingestion increased, the highest retentions, in all cases, occurred with the highest intakes. The average retentions of 147 and 169 units for the two highest ingestions of 224 and 311 units, respectively, are significantly greater than the average retentions of 12 and 27 units which accompanied the ingestions of 62 and 105 units. If the retentions based on the vitamin B of the food minus the total amount excreted in both urine and feces are considered the effect of the quantity of vitamin B in the diet is even more apparent, for retentions of 62 and 103 units were obtained with the higher levels of ingestion as compared to — 32 and — 14 for the two lower levels.

CHILD	DATE	ASSAY DATA FOR FOOD														
		Initial rat weight	Average dose per day	Gain of rat per day	Amount food for 1 unit vitamin B	Amount B per day	Initial rat weight	Average dose per day	Gain of rat per day	Amount urine for 1 unit vitamin B	Amount B per day	Initial rat weight	Average dose per day	Gain of rat per day	Amount feces for 1 unit vitamin B	Amount B per day
		gm.	gm.	gm.	gm.	units	gm.	cc.	gm.	cc.	gm.	gm.	gm.	gm.	units	
C.H.	11/23-25	66.5	4.0 <sup>1</sup>	....	....	208	63.5	1.00	1.46	0.57	61	129	0.43	1.87	63	
		60.5	4.0 <sup>1</sup>	....	....		67.0	0.98	1.60	0.61	57	166	0.28	1.45	63	
		78.5	4.0 <sup>1</sup>	....	....								0.83	0.90	33	
		73.5	5.0 <sup>1</sup>	....	....											
C.H.	11/14-17	64.0	2.2	1.40	1.57	208	90.5	0.65	2.08	0.31			0.77	0.95	33	
		73.5	1.9	1.10	1.68	195	57.0	0.63	2.41	0.24	166	65.5	0.62	1.50	69	
C.H.	11/18-21	64.0	2.2	1.40	1.57	208	66.5	0.60	1.50	0.40	100	58.0	0.59	0.85	31	
		73.5	1.9	1.10	1.68	195	63.5	0.55	1.14	0.44	91	64.5	0.40	1.55	81	
B.B.	1/16-20	68.5	3.7	0.50	4.7	64	65.0	1.19	1.17	1.1	45	45	0.60	1.40	36	
		54.5	3.4 <sup>1</sup>	....	....	73.5	1.31	1.00	1.3	38	72.0	0.56	1.15	31		
		59.0	4.0 <sup>1</sup>	....	....	69.0	1.82	1.43	0.9	56	70.5	0.69	1.45	32		
		66.0	4.5 <sup>1</sup>	....	....											
D.G.	2/25-3/1	53.0	2.9	0.80	3.63	81	55.5	0.66	0.78	0.85	71	53.5	0.55	1.22	36	
		65.5	3.2	0.83	3.80	77	63.0	1.17	1.12	1.04	58	70.5	0.57	1.17	34	
D.G.	11/23-25	62.0	3.1	1.00	3.54	83	66.5	0.74	1.30	0.57	61	71.0	0.66	1.55	21	
		64.0	2.4	1.00	2.4	122	53.5	0.76	1.15	0.66	53	66.0	0.54	1.30	21	
		59.0	3.5 <sup>1</sup>	....	....		70.0	1.21	1.95	0.69	51					
		69.5	3.4 <sup>1</sup>	....	....											
D.G.	1/16-20	74.0	4.0 <sup>1</sup>	....	....		63.5	0.53	0.88	0.6	83	69.0	0.51	1.55	47	
		76.0	3.0 <sup>1</sup>	....	....	57.5	1.00	1.35	0.7	71	64.5	0.51	1.21	40		
		65.5	5.0 <sup>1</sup>	....	....	68.5	1.40	1.35	1.0	50	73.0	0.42	1.06	39		
		51.5	4.0 <sup>1</sup>	....	....	68.0	1.10	1.20	0.9	56						
D.G.	2/ 5- 9	71.5	2.6	1.25	2.2	133	62.5	1.05	1.15	0.9	67	68.5	0.86	1.61	39	
		69.0	3.2	1.35	2.3	127	66.5	1.20	1.83	0.7	86	67.0	0.71	1.40	37	
D.G.	11/14-17	60.0	2.1	1.32	1.57	221	60.5	0.98	1.35	0.56	89	74.0	0.21	1.00	102	
		64.0	2.1	1.35	1.54	225	66.5	1.18	1.75	0.67	75	77.0	0.30	1.45	102	
D.G.	11/18-21	60.0	2.1	1.32	1.57	221	63.0	1.08	1.32	0.74	68	60.0	0.42	1.70	82	
		64.0	2.1	1.35	1.54	225	69.0	0.95	1.35	0.70	71	82.0	0.31	1.41	93	

J.F.	2/25-3/1	62.0	3.4	1.35	2.48	116	60.5	1.07	1.25	0.86	70	73.0	0.40	1.18	0.34	41
		64.0	3.0	1.00	2.54	113	67.0	1.27	1.04	0.91	66	70.5	0.45	1.40	0.32	43
		73.5	1.6	0.61	2.60	111										
F.V.	11/23-25	61.0	3.0 <sup>1</sup>	....	....		66.0	1.33	1.13	1.18	30	84.0	0.42	1.30	0.32	51
		73.5	3.0 <sup>1</sup>	....	....		73.5	1.21	0.90	1.21	29	68.0	0.36	1.22	0.29	56
		52.5	3.0 <sup>1</sup>	....	....											
		66.5	5.0 <sup>1</sup>	....	....											
F.V.	1/16-20	69.0	2.5	0.50	4.7	63	67.5	0.84	0.79	1.0	50	67.0	0.65	1.23	0.48	41
		65.0	3.5	1.25	4.8	62	66.5	0.97	0.89	1.1	45	82.5	0.28	0.96	0.29	68
		67.0	4.5	....	....		78.0	0.98	0.80	1.2	42	75.5	0.34	1.00	0.34	58
F.V.	12/10-14	68.0	2.7	0.75	3.5	97	71.0	0.76	1.20	0.6	83	61.0	0.54	1.95	0.28	85
		57.0	3.7	0.79	3.8	89	59.0	0.68	1.40	0.5	100	65.5	0.56	1.23	0.24	99
		61.0	4.5	....	....											
F.V.	2/ 5- 9	72.0	2.6	0.80	3.3	85	67.0	0.96	1.27	0.7	86	78.0	0.62	1.50	0.41	47
		70.5	3.5	0.94	3.7	76	87.0	1.03	1.78	0.6	100	74.5	0.77	1.45	0.53	37
		70.0	4.0	1.30	3.1	90	65.5	0.74	1.45	0.5	120	82.0	0.57	1.25	0.46	42
F.V.	11/14-17	63.0	1.6	1.08	1.39	244	67.0	1.00	0.75	1.13	44	63.5	0.41	1.25	0.33	66
		70.0	1.9	1.35	1.37	247	59.5	1.35	1.00	1.34	37	77.0	0.25	1.65	0.15	146
												70.5	0.28	1.15	0.24	91
F.V.	11/18-21	63.0	1.6	1.08	1.39	244	60.0	0.76	2.11	0.36	37	62.5	0.53	1.50	0.27	91
		70.0	1.9	1.35	1.37	247	66.0	0.75	1.95	0.38	36	62.5	0.31	1.32	0.21	117
							61.0	0.64	1.60	0.40	36	57.0	0.22	1.40	0.16	154
J.S.	3/15-19	56.0	2.3	2.11	1.08	324	65.5	0.76	1.91	0.40	125	52.0	0.47	1.85	0.25	97
		68.0	1.9	1.75	1.05	333	66.0	0.64	1.45	0.40	125	62.0	0.58	1.80	0.32	76
J.S.	3/20-24	80.0	2.5	1.46	1.53	229										
		68.0	1.7	1.35	1.22	287	54.0	0.66	1.71	0.37	138	57.0	0.22	1.70	0.13	180
		73.0	2.0	1.67	1.31	289						69.0	0.31	1.70	0.18	130
		57.5	2.3	1.41	1.60	219	58.5	0.48	1.40	0.34	150					
P.D.	3/15-19	Not assayed See results of similar food for period 3/20-24					72.0	0.73	1.50	0.49	104	72.0	0.83	1.63	0.51	53
												69.5	0.91	1.40	0.65	42
P.D.	3/20-24	68.0	1.7	1.41	1.18	275	80.0	0.69	1.59	0.44	123					
		73.5	2.1	1.95	0.95	341	54.5	0.62	1.77	0.32	168	67.0	0.53	0.85	0.62	43
							67.5	0.45	1.30	0.35	154	71.0	0.49	1.15	0.43	61
V.M.	3/15-19	65.0	1.9	2.10	0.91	373	70.0	0.59	1.33	0.44	125	58.5	0.66	2.70	0.24	92
							60.5	0.74	1.65	0.45	122	62.0	0.42	2.15	0.20	110
V.M.	3/20-24	67.0	1.8	2.46	0.74	459	65.0	0.56	1.95	0.29	190	74.5	0.57	1.70	0.34	59
		62.0	2.1	2.40	0.88	386	70.0	0.49	1.64	0.27	204	66.5	0.30	1.50	0.20	100
							74.5	0.47	1.67	0.34	229	70.0	0.36	1.75	0.23	87

<sup>1</sup> For explanation of these periods see text, paragraph 2 of 'Discussion of Results.'

Both of the methods of calculating vitamin B retentions (food minus urine and food minus total excretion) have been employed in this study since our knowledge of the metabolism of vitamin B is too limited at the present time to permit a

TABLE 3

*The vitamin B intakes, excretions and retentions of the individual children for each period, in units of vitamin B per day*

CHILD	DATE	AGE	WEIGHT	VITAMIN B				
				Food	Urine	Feces	Retention	
							Food-urine	Food-total excretion
		<i>yr.—mo.</i>	<i>kg.</i>	<i>units</i>	<i>units</i>	<i>units</i>	<i>units</i>	<i>units</i>
C.H.	11/23-25	4-9	16.3	60 <sup>1</sup>	59	53	1	—52
C.H.	11/14-17	4-9	15.8	202	137	48	65	17
C.H.	11/18-21	4-9	15.8	202	96	48	106	58
B.B.	1/16-20	4-2	15.4	64	46	33	18	—15
D.G.	11/23-25	4-8	19.3	60 <sup>1</sup>	55	21	5	—16
D.G.	1/16-20	4-10	19.8	63 <sup>1</sup>	65	42	—2	—44
D.G.	2/25-3/1	4-11	19.9	91	65	38	26	—12
D.G.	2/ 5- 9	4-10	19.8	130	77	38	53	15
D.G.	11/14-17	4-8	19.3	223	82	102	141	39
D.G.	11/18-21	4-8	19.3	223	70	89	153	64
J.F.	2/25-3/1	4-2	19.0	113	68	42	45	3
F.V.	11/23-25	3-11	16.1	60 <sup>1</sup>	30	54	30	—24
F.V.	1/16-20	4-1	16.4	63	46	56	17	—39
F.V.	2/ 5- 9	4-2	16.8	84	102	42	—18	—60
F.V.	12/10-14	4-0	16.6	93	92	92	1	—91
F.V.	11/14-17	3-11	15.9	246	41	101	205	104
F.V.	11/18-21	3-11	15.9	246	36	121	210	89
J.S.	3/15-19	6-9	21.5	328	125	87	203	116
J.S.	3/20-24	6-9	21.5	256	144	155	112	—43
P.D.	3/15-19	4-8	15.8	308	104	48	204	156
P.D.	3/20-24	4-8	15.8	308	148	52	160	108
V.M.	3/15-19	5-3	21.1	373	124	101	249	148
V.M.	3/20-24	5-3	21.1	422	208	82	214	132

<sup>1</sup> Estimated.

complete explanation of the significance of vitamin B in the urine and feces. The vitamin B excreted in the urine cannot be entirely a surplus of intake above requirement since vitamin B is present in appreciable amounts in urine during low levels of ingestion and furthermore the amount in the urine

does not increase comparably to food increases when the level of ingestion is raised. The vitamin B of the feces may be partially unabsorbed residues from the food, but similarly to the findings with urine, the quantity in the feces does not increase proportionately to increases in food intake. In addition, it is apparent that a relation between the excretion

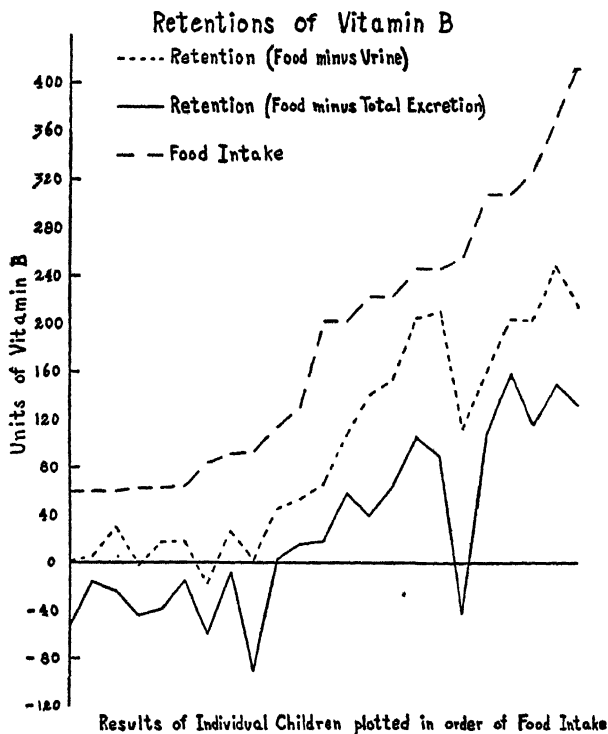


Figure 1

of vitamin B in the feces and the urine may exist since there was a trend in this study for feces figures to be high when urine was low and vice versa. Further work is now in progress to learn more regarding vitamin B metabolism and an attempt is being made to study the factors influencing the excretion of vitamin B in urine and feces.

Gaynor and Dennett ('34) have reported that their data for infants indicate but little storage of vitamin B in the body. A similar conclusion may be reached from analysis of the retentions for D.G., F.V., and C.H., 11/23-25. Although high ingestion periods (11/14-17, 11/18-21) preceded the period 11/23-25 by only one day, the three children showed an average retention of only 12 units when calculated on the basis of food-urine and an average negative balance of — 31

TABLE 4  
*Average vitamin B values for each level of vitamin B ingestion, in units of vitamin B per day*

NUMBER OF CHILDREN	VITAMIN B										
	Food		Urine		Feces		RETENTION				
	Range	Aver- age	Range	Aver- age	Range	Aver- age	Food-urine		Food-total excretion		
							Range	Average	Range	Average	
6	60 64		30 65	50	21 56	43	— 2 — 30		— 52 — 15		— 32
1		93		92		92		1			— 91
4	84 130		65 102		38 42		— 18 — 53		— 60 15		— 14
6	202 246		36 137		48 121		65 210		17 104		62
6	256 422		104 208		48 155		112 249		— 43 156		103

units when total excretion was considered. Thus the average total retention of 62 units for the preceding period was not sufficient to counteract the low ingestion of period 11/23-25. It would seem, therefore, that excess supplies of vitamin B are quickly exhausted from the body.

In view of this apparent inability of the body to build up a reserve of vitamin B, the need for an adequate supply is particularly important. As an aid in determining how much is required for an optimum intake, the vitamin B contents of food, urine and feces have been calculated on a per kilogram

basis for each child and plotted, in order of amount ingested, in figure 2. It will be noted that ingestions were not consistently above the total quantity of vitamin B excreted until more than 12 units per kilogram were included in the daily allowance. The highest retentions were obtained with an ingestion of 20 units of vitamin B per kilogram. Since retentions consistently continued to increase with increased ingestions, the tentative conclusion is advanced that at least 20 units

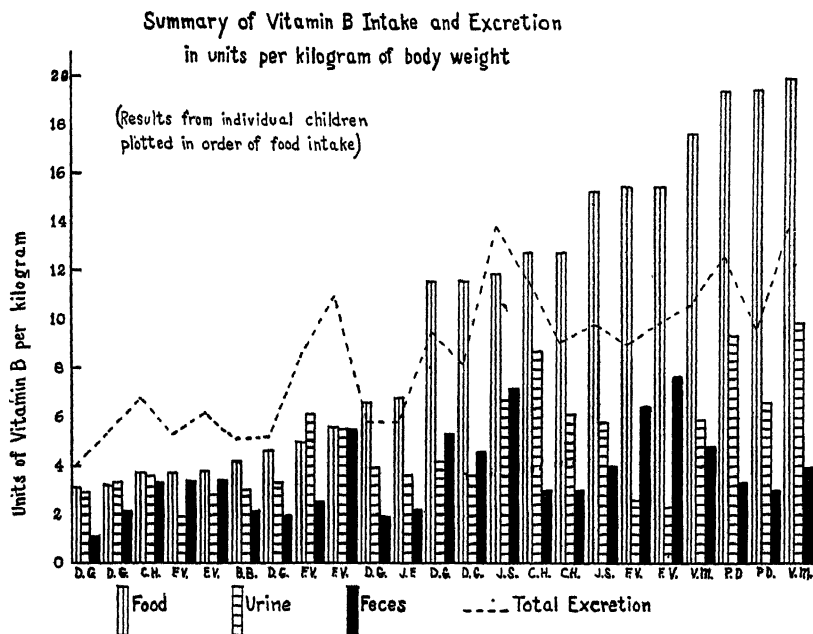


Figure 2

of vitamin B per kilogram of body weight are necessary each day as a satisfactory intake to insure optimum health. In terms of Chase-Sherman units, this intake is approximately equivalent to 40 units per kilogram per day.

This high vitamin B requirement is comparable to the recent findings of Waterman and Ammerman ('35) with purified vitamin B. These authors state: "It should be noted that the upper limit of beneficial increment of the vitamin has not been reached although the vitamin intake which can generally

be secured by a choice of natural foods has apparently been surpassed." In the present study, the intakes which resulted in the highest retentions (regardless of whether retentions were calculated on the basis of food minus urine or food minus total excretion) were found to be six to seven times greater than the minimum requirement for preventing beri-beri as determined by means of the formula suggested by Cowgill ('34).

If the intakes resulting in highest retentions may be considered optimum, this wide range between minimum and optimum requirement would seem to explain both the existence of vitamin B deficiency among children and the beneficial results obtained by additions of vitamin B to the diet.

#### SUMMARY

The utilization of vitamin B, as affected by various levels of ingestion has been studied by comparing the retentions of eight young children during twenty-three metabolism periods.

The dried food aliquots, dried feces, and concentrated urine from the children were assayed for their vitamin B content according to standardized technic. The unit of vitamin B selected as being most suitable to the short period type of assay employed was the quantity of vitamin B causing 1 gm. of gain. This unit is approximately equal to 2 Chase-Sherman units.

The outstanding result of the investigation was the fact that increasingly higher retentions were obtained with higher intakes for each level of ingestion studied.

On the basis of the ingestions giving the highest retentions, the optimum requirement of vitamin B by young children is estimated to be 20 units per kilogram of body weight, or about 40 Chase-Sherman units per kilogram per day.

The writer wishes to take this opportunity to thank Dr. Amy L. Daniels, whose help and advice made this study possible, and also to thank members of the nutrition staff of the Iowa Child Welfare Research Station for their cooperation.



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# THE SPECIFIC DYNAMIC ACTION OF BUTTER FAT, AND OF SUPERIMPOSED SUGAR

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SIX FIGURES

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In a previous paper from this laboratory by Hawley, Johnson and Murlin ('33) on the possibility of gluconeogenesis from fat in the human subject, some data relative to the specific dynamic effect of the high fat (cream) meals were presented. A more complete treatment of this phase of high fat metabolism was reserved for study with the new respiration calorimeter (Murlin and Burton, '35). The purpose of using the calorimeter for this study was to obtain, if possible, reliable measurements of the heat value of a liter of oxygen at respiratory quotients below the level for combustion of fat by making use of the direct heat. It was believed that heat values found by extrapolation could be fairly checked by that means. This would make possible the construction of dynamic curves from which the total effect of a given meal could be deduced, even though the R.Q. should fall considerably below 0.707. The interpretation of these low R.Q.'s should be more secure once the heat relations were understood.

## EXPERIMENTAL

All the work has been done on nine male subjects, all but one of them members of the department as staff or students.

They ranged in age from 23 to 61 years and in weight from 68 to 94 kg. Several of the experiments represent the subject's first experience on high fat and several exhibit the signs of improved tolerance on repetition of the diet which were discussed in the earlier report.

The calorimeter was supplemented in the indirect determination of heat production by the Tissot-Haldane method. Having a particularly efficient Haldane air analyzer, designed by Dr. E. S. Nasset, it has not been unduly burdensome either to observer or subject to superimpose the short 10-minute periods with the Tissot, thereby completing the record without keeping the subject confined too long. In the tables following, *T* just before the time of period stands for the Tissot-Haldane and *C* for the calorimeter. It has been gratifying to find that the two totally different types of determination of indirect heat often agreed, where they were supposed to agree, quite as well as could be expected (e.g., table 6, June 1; table 7, June 22). Candor requires the further statement, however, that in some instances (e.g., table 1, January 16) the agreement is not too good. Such differences are attributable to variations in temperature or to the discomfort sometimes caused by a nose clip.

In some of the later experiments, for convenience in attaching the surface thermometers (Burton, '34, '35) the subject entered the calorimeter nude except for a pair of 'shorts,' and yet the basal in this condition, when the temperature was equally comfortable, duplicated the basal taken by the spirometer method when the subject was fully clothed (e.g., table 6, June 1). When there was a difference of as much as 3 or 4° between room temperature and calorimeter temperature, the agreement was not so good. However, with a subject possessing a liberal amount of subcutaneous fat this difference in temperature did not increase the metabolism (table 4, June 13) when he was nude.

*Check experiments*

Numerous heat checks have been run during the period, from June 1933 to July, 1936, within which these experiments were made, in order to be assured that the calorimeter was operating correctly. On recalibration for heat loss through the wall (Murlin and Burton, '35) after the summer vacation of 1934 a slightly different calibration curve was obtained and for the last six experiments, done since that time, the new curve has been used.

Altogether forty-five alcohol check periods were run from May, 1933 to June, 1936, at times adjacent to the experiments. The average R.Q. obtained was 0.663 with an average deviation from the mean of  $\pm 0.018$ . The average difference between direct and indirect heat in individual periods was 3.6%,<sup>1</sup> the algebraic mean  $+ 1.69\%$  or 1.3 Cal. The direct heat was the higher. The agreement between different respiratory quotients from the same expired air with the Tissot-Haldane method is better than  $\pm 0.01$ .

*Calculation of indirect heat for R.Q.'s below 0.707*

While accumulating data on the heat value of a liter of oxygen for low R.Q.'s by the direct measurement, it was necessary meantime to compare direct and indirect heats for evaluation of the specific dynamic action. Inspection of the low quotients in the tables reveals that in comparison with other periods with higher quotients, they are due in most instances to high oxygen values rather than to low CO<sub>2</sub> values. This is best seen in basal periods on the Tissot (e.g., tables 1 and 2). It has been customary in many laboratories to calculate heat production for non-protein R.Q.'s less than 0.707 as if the quotient were 0.707, thereby admitting error in the determination and closing the mind to the possibility of uses of oxygen other than for combustion. While it is true that in the closed circuit method there are more chances of error on oxygen than on CO<sub>2</sub>, with frequent alcohol checks it is not necessary

<sup>1</sup> Exactly the same whether calculated on CO<sub>2</sub> or O<sub>2</sub>.

to concede serious error (see check experiments). With the growing evidence that  $O_2$  may be used for other purposes, it is conservative to regard the  $CO_2$  as correct and to base the calculation of indirect heat on the assumption that all of the non-protein  $CO_2$  represents combustion of fat in these low quotients. If the  $CO_2$  is correct and the extra  $O_2$  which is responsible for the low R.Q. is used for some other purpose than combustion, the heat production on the basis of  $CO_2$  at an R.Q. of 0.707 and the heat production on the basis of  $O_2$  at the value ( $\frac{4.686 \times \text{low R.Q.}}{0.707}$ ) will be the same. This is shown in the footnote.<sup>2</sup>

Either method of calculation obviously gives a lower value than to base the calculations on the oxygen at the non-protein R.Q. of 0.707. In other words, the assumption is made that the extra oxygen which gives the low R.Q. is being used entirely, as we know from the ketosis that it is being used in part, for some purpose other than complete combustion. Furthermore, this procedure gives better agreement with the direct measurement in most cases,<sup>2</sup> as Adams and Poulton<sup>3</sup> ('36) have shown is true on the basis of the data of DuBois. Having adopted this method of calculation for low quotients obtained with the calorimeter, it was necessary to adopt it also for the Tissot periods, even though the oxygen determinations with this method are just as reliable as the  $CO_2$ .

A few comparisons are possible between the heat values for a liter of oxygen obtained in this way with the values

<sup>2</sup>For example the following comparisons may be cited:

Non-protein R.Q.	Indirect heat calculated on		Extrapolation 4.686 Cal. / low N.P. R.Q. 0.707 Cal.	Direct heat
	1 $O_2$ at non- protein R.Q. of 0.707 Cal.	2 $CO_2$ at non- protein R.Q. of 0.707 Cal.		
0.60	107.2	93.7	94.3	84.6
0.61	99.2	87.1	87.0	91.5
0.61	100.0	92.0	91.8	96.8
0.65	80.8	78.7	78.7	76.0
0.68	88.8	85.1	85.2	78.3
0.689	78.3	74.5	74.5	74.5
0.695	100.1	98.7	98.6	98.0

<sup>3</sup>Our data had been assembled and tabulated as above before we were aware of the work of Adams and Poulton.

obtained from direct heat, by subtracting protein calories and dividing by the liters of non-protein oxygen. Thus,

Non-prot. R.Q.	Heat value of a liter of O <sub>2</sub>	
	$\frac{4.686 \times \text{low R.Q.}}{0.707}$	$\frac{\text{Direct heat—prot.Cal.}}{\text{Non-prot. O}_2}$
0.60	3.976 Cal.	3.521 Cal.
0.61	4.043	4.281
0.64	4.241	4.355
0.65	4.308	4.355
0.675	4.292	4.473
0.68	4.507	4.277
0.686	4.546	4.566
0.69	4.573	4.572

There is not yet in the collection of data a sufficient number of calorimeter periods with low R.Q.'s and determination of the nitrogen in the urine to extend the comparison further. The agreement between the two methods depends upon the errors of direct heat measurement and those implicit in the protein heat. With perfect measurement and evaluation of these two factors oxygen values necessarily agree.

The mean indirect heat for the total of forty-two periods with these subjects is  $83.01 \pm 0.85$  Cal. and for the direct heat  $83.31 \pm 0.88$  Cal. per hour, a difference of only 0.3 Cal. or 0.36% of the indirect.<sup>4</sup> The real test of agreement in any particular period, however, is the mean of the individual differences, which is  $\pm 3.28$  Cal. or 3.95%, practically the same as the alcohol checks. Only one other series of heat measurements on human subjects employing both direct and indirect calorimetry in hourly periods ever has been reported in which the percentage difference between the two means was less than the 0.36% recorded here (Gephart and DuBois, '15).

<sup>4</sup> The significance ratio as to the differences of the means is found by comparing the difference, 0.30 Cal., with its probable error. Unless it is three times the probable error of the difference the difference itself is usually regarded as insignificant. The P.E. of the difference of means is found by the formula  $\sqrt{E_1^2 + E_2^2}$  (Davenport, '04) where  $E_1$  and  $E_2$  are the probable errors of the two means, 83.01 and 83.31. The formula  $0.6745 \frac{\sigma}{\sqrt{n}}$  gives  $E$ . The standard deviations are 8.17 and 8.51 Cal., respectively. Therefore the P.E. of the difference of the means is 1.22 and the ratio 0.25; which is to say that in two such series of measurements of the same quantity, a difference of 0.3 Cal. shows that one method (direct) is just as dependable as the other (indirect).

The authors reported a difference between their totals for 61 hours of 0.17%. Their series, however, contains only fifty-four periods<sup>5</sup> in which figures are correctly given for both indirect and direct measurements on the hourly basis. The difference of the means in these fifty-four periods is 0.25 Cal. or 0.324% of indirect. The mean difference between the two methods is  $\pm 4.49$  Cal. or 5.8% of indirect. It is evident that the present series, shorter by ten periods, shows a slightly better agreement in the average hourly determination than that of Gephart and DuBois, notwithstanding their slightly better agreement between totals.

It is gratifying to find that the agreement between direct and indirect heat improves a little as more experience with the calorimeter is gained. In describing the calorimeter (Murlin and Burton, '35) it was stated (p. 258) that the average percentage difference in individual periods was of the order of 7 or 8%, but that by use of the surface thermometers together with the rectal to indicate change of body temperature, the difference had been brought down to  $\pm 5.5\%$ . None of the first four experiments of the present series was among those in which the improved method of securing change of body temperature was adopted. With five of the later subjects (V to IX inclusive) the surface temperatures were measured; but in one (VII) because of an accident to the chest thermometer, they could not be used. In only 5 of the calorimeter days out of the 18 here reported therefore was any use made of the surface temperatures. Rectal temperature, of course, was used in all. It is hoped that still better agreement may be attained by use of the surface thermometers in future work. The improvement in precision, however, is due mainly to the use of  $\text{CO}_2$  as a basis for calculation of the indirect heat for low R.Q.'s as described above.

<sup>5</sup> There are several obvious errors of addition in their table 3. These are excluded as well as the results given in two hourly determinations.



## PROCEDURE

Certain features of the daily program on experimental days will be apparent from the representative tables. Following in a general way the dietary procedure of a former study (Hawley, Johnson and Murlin, '33) for the highest fat intake (F.A. to G ratio of 4.1), heavy (4X) cream in amount necessary to cover the 24-hour caloric requirements was taken. The only adjuvants permitted were lettuce and coffee, and the lettuce was generally omitted on calorimeter days. It served the purpose mainly of affording something to masticate and to cleanse the mouth of the creamy after-taste. The coffee, sweetened occasionally with saccharine, afforded a vehicle for more agreeable ingestion of a large part of the cream. The lettuce rarely exceeded 100 gm. at a meal and the coffee two cups. As a rule the subject ingested the all-cream diet for a total period of at least 5 days. Probably no other diet supplying practically all the energy from fat can be tolerated equally well for so long a period. According to the earlier experience the lowest R.Q.'s were obtained only after the second day. Exploratory determinations therefore were made on the third day of the diet, with the Tissot-Haldane method, and in several instances the calorimeter was used to get a basal determination. The fourth day of the diet was expected to be the most favorable for low quotients, but it did not turn out so in all cases. After further exploratory determinations in post-absorptive conditions on this day, the subject then took a large meal of fat (the largest he could tolerate) and entered the respiration calorimeter for the double determination of the heat production for several hours. Some twenty subjects have been studied in the calorimeter to date and all but two or three have found the bed and surroundings comfortable for a total of 3 to 4 hours. Practically complete muscular repose can be maintained.

## CALCULATION OF S. D. A.

Specific dynamic action of the food, as the term is used in this paper, refers to the increase in heat production over post-absorptive basal. Of the several known ways of expressing the specific dynamic effect of food, that one originated by Benedict and Carpenter ('18) which includes the entire area between the base line and the curve of excess and expression in terms of calories fed seems to the present writers to be the most suitable for study of ordinary foodstuffs. Where food substances of known molecular weight in crystalline purity can be given, doubtless the expression in terms of mols (Wilhelmj, '34) is to be preferred. Percentage increase over basal is also given in the individual tables.

Construction of the complete curve of excess over basal must of necessity be a piecemeal procedure for a calorimeter. Even if the subject could endure a continuous experiment for 10 successive hours and maintain complete repose, the observers would find it exceedingly laborious. By starting at different intervals after feeding meat and running a few hours each day, Williams, Riche and Lusk ('12) were able to follow its dynamic effect in the dog to completion, and the same method was used by Murlin and Lusk ('15) for the dynamic action of fat in the dog. The purpose of the present study made it impossible to follow this method systematically, but a sufficient number of points on the curve was obtained to draw a plausibly complete curve for all but one subject. The curves will be described in the next section.

## RESULTS

It will not be possible, because of limitation of space, to present all the results except in summary. Three experiments on as many subjects taking high fat alone were done in June and July, 1933 when the weather was quite hot. Subject I had been on the all-cream diet previously in 1930 and 1931, but it may be supposed that any special adaptation to high fat acquired at that time had been lost in the 2 years and 5 months intervening. It is also possible that the hot weather made

the fat diet less tolerable, in the sense of less perfect combustion, for all three subjects. Subject II had not had previous experience with the diet; he exhibited a very low tolerance. Subject III had taken it for 5 days 2 weeks earlier in July and had exhibited many more low respiratory quotients than he did in the later experiment. There is evidence of adaptation therefore in his case (Hawley, Johnson and Murlin, '33). The subject who had lost any special adaptation to the metabolism of high fat showed quite low quotients in the basal periods as well as in the calorimeter.

The heavy cream used (38 to 41% ether extract) contained all but about 5% of the calories from fat (Sherman, '33). Subjects I and II ingested as a test meal 450 gm. with a small amount of lettuce which would supply not more than 5 gm. carbohydrate. The specific dynamic action of the 171 gm. fat in the case of subject I reached on the first day the level of 20.3% above basal at about the third hour (time always calculated to middle of the respiratory or calorimetric period). The next day at about  $\frac{1}{2}$  hour earlier, it rose to 24.6%. These figures compare favorably with the value of 23% obtained by Murlin and Lusk ('15) in a dog after ingestion of 75 gm. emulsified peanut and lard oils mixed. Subject II in his highest period gained a level of only 16.2%, which probably is explained by poor digestion. In fact this subject was so distressed by the high fat that he was obliged to discontinue the experiment the next morning after the first basal period. Subject III endured the diet very well after the previous adjustment period 2 weeks earlier, and on the fifth day produced a dynamic effect from 152 gm. fat which at the high point was 27% above basal. The basal periods, however, were obtained the day before. Eighty grams fat with this subject in corresponding periods the first day gave a dynamic effect a little more than half as great as after 152 gm. the second day. To turn the comparison about, a dose 1.9 times as great produced a dynamic effect 1.8 times as great as the first day. Digestion and absorption obviously were satisfactory.

Three experiments were done in January, 1934 (illustrated by tables 1 and 2) in which two important modifications from the previous experiments were made. First, after the calorimetric study on the fourth day of the high fat diet, a large dose of sugar was taken and the study of dynamic effect was continued by the Tissot-Haldane procedure. The same dose was repeated after the basal periods on the fifth day. R.Q.'s and S.D.A. of the superimposed sugar could now be compared in the two conditions. Subjects I and II were the same persons of corresponding numbers as in the earlier experiments. Probably because of the time of year and almost certainly because both subjects 6 months before had become at least partially adapted (II) and readapted (I), respectively, they tolerated the diet much better. At all events they metabolized the fat more completely, as judged by the R.Q.'s. Secondly, also the ketosis was followed by analysis of the blood and urine for ketone bodies. Subject I following a somewhat larger meal of fat, had shown a ketosis (table 3) of approximately the same order in 1931 (Hawley, Johnson and Murlin, '33, p. 535) and R.Q.'s within the same range.

Subject IV was having his first experience with high fat (table 2). He was also the youngest of the subjects and a rather stout, but not quite obese, person. Although the test meal of fat taken on the fourth day of the diet was decidedly smaller than that taken by subject I, the resulting ketosis of subject IV was considerably higher (table 3). Also the basal ketosis was the highest on each of the three days (third, fourth and fifth of the diet) shown by any of the subjects. We are prepared therefore for lower R.Q.'s in this subject. He had all the signs except alimentary distress of low tolerance for fat. In the second calorimeter period, the non-protein R.Q. reached the low point of 0.61, which is far too low to be accounted for by any degree of ketosis and possible formation of sugar from glycerol and protein (Hawley, Johnson and Murlin, '33, p. 554). It should be observed also that the basal R.Q.'s by the Tissot-Haldane method on the third and fourth days of the diet were quite low.

TABLE 1  
Specific dynamic action of butter fat and of sucrose superimposed (subject II)

DATE 1934 JANUARY	TIME <sup>5</sup> AFTER SUGAR	TIME <sup>5</sup> AFTER FAT MEAL	APPLIANCE	Period	CO <sub>2</sub>	O <sub>2</sub>	R.Q.	N./HR.	NON- PROTEIN R.Q.	HEAT CAL./HR. Indirect	Direct	S.D.A. PER CENT OF INDIRECT
16 <sup>1</sup>	minutes	minutes			L./hr.	L./hr.		gm.		73.2 <sup>2</sup>		
	Basal	Basal	T	8.50-9.00 A.M.	11.37	15.92	0.71	0.432	0.70			
			T	9.08-9.18 A.M.	11.04	15.53	0.71	0.432	0.69	71.0 <sup>4</sup>		
			C	11.15-12.16 P.M.	11.66	16.03	0.73	0.412	0.71	74.6	78.5	
17					In calorimeter—no food							
			T	8.53-9.03 A.M.	10.85	14.85	0.72	0.490	0.71	69.0		
			T	9.09-9.19 A.M.	10.95	13.90	0.79	0.490	0.78	65.5		
				9.40 Took 240 gm. 4X cream = 91 gm. fat.	In calorimeter							
18		91	C	10.51-11.52 A.M.	13.15	17.41	0.75	0.508	0.74	81.6	83.0	21.4 <sup>3</sup>
		161	C	11.52-12.51 P.M.	12.72	16.89	0.75	0.508	0.74	79.1	76.3	13.5
					In calorimeter					160.7	159.3	
		52	T	1.23 Took 100 gm. sucrose								
		308	T	2.10-2.20 P.M.	15.29	18.38	0.83	0.487	0.84	88.0		30.8
		130	T	2.43-2.53 P.M.	15.21	17.18	0.88	0.487	0.90	83.3		24.1
			T	3.28-3.38 P.M.	13.6	16.22	0.84	0.487	0.85	77.7		15.6
		Basal	T	8.49-8.59 A.M.	9.96	15.33	0.65	0.234	0.63	64.9 <sup>3</sup>		
		Basal	T	9.06-9.16 A.M.	10.51	14.11	0.74	0.234	0.74	65.6		
				9.45 A.M.	Took 100 gm. sucrose							
		53	T	10.33-10.43 A.M.	14.07	16.34	0.86	0.399	0.87	78.8		20.1 <sup>4</sup>
		83	T	11.03-11.13 A.M.	14.56	17.00	0.86	0.399	0.86	82.0		25.0
		130	T	11.50-12.00 M	13.80	16.46	0.84	0.399	0.84	78.9		20.2

<sup>1</sup> Third day of all-cream diet.

<sup>2</sup> Calculated on non-protein R.Q. found, by extrapolation from 0.707.

<sup>3</sup> Using 67.2 Cal./hr. mean of indirect as basal.

<sup>4</sup> Using 65.6 Cal./hr. mean of indirect as basal.

<sup>5</sup> *mean value of experimental period.*

TABLE 2  
*Specific dynamic action of butter fat and of glucose superimposed (subject IV)*

DATE AFTER 1934	TIMES AFTER SUGAR	TIMES AFTER FAT MEAL	APPLIANCE	RESPIRATORY EXCHANGE						HEAT CAL./HR.		S.D.A. PER CENT OF INDIRECT
				Period	CO <sub>2</sub>	O <sub>2</sub>	R.Q.	N./hr.	Non- protein R.Q.	Indirect	Direct	
January 30 <sup>1</sup>	minutes	minutes	T	8.52- 9.02 A.M.	L./hr. 12.87	18.99	0.63	0.506	0.66	83.4 <sup>2</sup>		
		Basal	T	9.14- 9.24 A.M.	12.43	17.62	0.70	0.506	0.69	80.1		
	Basal	Basal	T	8.52- 9.02 A.M.	12.86	18.02	0.71	0.518	0.69	82.9 <sup>2</sup>		
		Basal	T	9.13- 9.23 A.M.	13.12	18.90	0.69	0.581	0.67	84.0		
February 1	minutes	minutes	C	10.05 A.M.	Took 250 gm. 4X cream = 95 gm. fat							
		91	C	10.30 A.M.	In calorimeter							
			C	11.12-12.00 A.M.	13.90	19.83	0.70	0.672	0.67	88.6 <sup>2</sup>	96.3	6.1 <sup>2</sup>
		140	C	12.00-12.49 P.M.	14.40	22.30	0.65	0.672	0.61	93.0 <sup>2</sup>	96.8	10.1
			T	1.30 P.M.	Took 100 gm. glucose							
		52	T	2.19- 2.29 P.M.	15.87	21.51	0.74	0.858	0.72	99.9		19.7
			T	2.52- 3.02 P.M.	16.02	21.38	0.75	0.858	0.74	99.7		19.5
		87	T	3.52- 4.03 P.M.	14.57	18.89	0.73	0.858	0.708	92.1		10.3
			T	8.46- 8.56 A.M.	12.37	17.17	0.72	0.519	0.702	79.8		
		147	T	9.08- 9.18 A.M.	12.33	16.95	0.72	0.519	0.71	78.7		
			T	9.45 A.M.	Took 100 gm. glucose							
		54	T	10.34-10.44 A.M.	14.80	19.20	0.77	0.673	0.76	90.1		13.6 <sup>4</sup>
			T	11.13-11.23 A.M.	15.00	18.72	0.80	0.673	0.80	88.5		11.7
		93	T	12.08-12.18 P.M.	15.42	18.54	0.83	0.673	0.84	88.4		11.5
	148		T									

<sup>1</sup> Third day of all-cream diet.

<sup>2</sup> Calculated on heat value of non-protein R.Q. found, by extrapolation from 0.707.

<sup>3</sup> Using 83.5 Cal./hr. mean of indirect as basal.

<sup>4</sup> Using 79.3 Cal./hr. mean of indirect as basal.

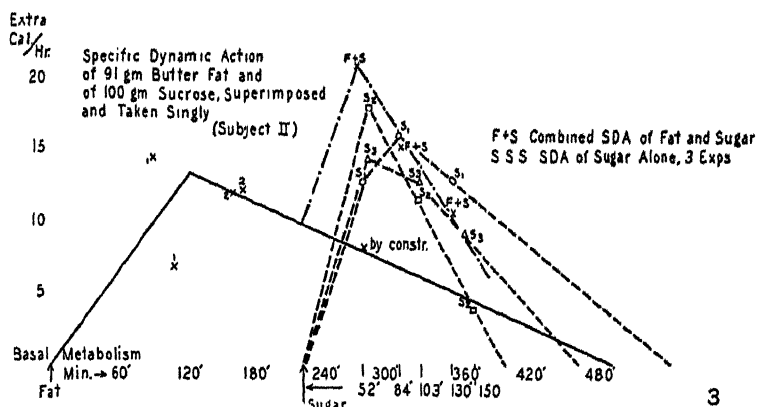
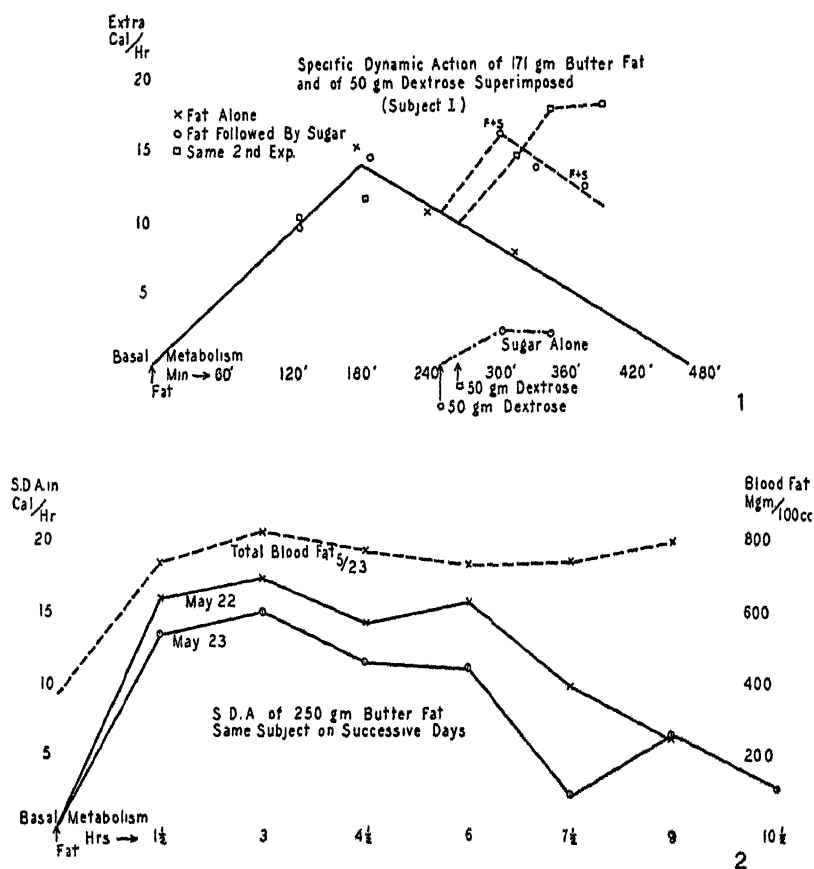
<sup>5</sup> To middle of experimental period.

T = Tissot-Haldane method; C = calorimeter.

Just as with subject II in his first experiment this subject (IV) with very low tolerance exhibits a rather low specific dynamic action of the fat—only 6.1% at 1½ hours and 10.1% at 2½ hours. Subject II in the adapted state (table 1), however, showed by contrast a marked S.D.A., namely 21.4%, at 1½ hours. It falls rather abruptly, it is true, in the next period, but even if we take the mean which is 17%, we find it more than double that of the corresponding figure in his earlier experiment. This also betokens better utilization.

The contrasting effects of fat to raise the heat production in these three subjects are best shown graphically (figs. 1, 2 and 3). Figure 1 is a combined graph of four different experiments on subject I, who took in each case 450 gm. of the heavy cream, containing 171 gm. butter fat. Figure 2 shows two additional curves for this subject taken from experiments made on May 22 and 23, 1930 and reported incompletely by Hawley, Johnson and Murlin ('33). The amount of fat taken at the test meal was about 80 gm. more than in the recent experiments. The relation to the blood fat taken every 1½ hours from the meal time on one of these days (May 23) is shown. The difference between the dynamic curves is more than accounted for by the difference in basal levels from which the curves are measured. In other words, if the earlier basal had been used the curves would be reversed. This may signify, as was intimated in the earlier report, that there is a true *luxus* consumption contained in the higher basal on the second day (fifth of the diet); but there is no confirmatory evidence in the present study. There was no excess of calories for 24 hours in the all-cream diet and, as shown by nearly every table in this paper, the basal heat production on successive days is quite uniform, or falls slightly. The latter effect, however, usually comes after sugar had been taken.

The relation to total blood fat is of interest because it shows that the S.D.A. does not run parallel to it except in a rather rough way up to the sixth hour. Beyond this point blood fat rises after the dynamic effect has fallen sharply. The S.D.A.



Figures 1 to 3



of fat therefore does not appear to be due entirely to plethora in the sense of Lusk.

All the S.D.A. curves show that the rise is much more abrupt than the decline, and there is a sort of family resemblance in the general proportions of all of them.

Figure 1 is the best authenticated curve, for it is a composite of four experiments all of which gave points which agreed well. Figure 3, on the contrary, is averaged graphically from two experiments which were not very concordant and one point on the down slope was obtained 'by construction,' i.e., by subtracting the average effect of three sugar experiments from the effect of fat plus the same amount of sugar in one. The resulting curve is too high (table 8) and is included only for the sake of showing the comparison between the superimposed sugar curve and the three curves after sugar alone, which is discussed below.

The third group of experiments carried out in May and June, 1935, were identical in procedure with the preceding group. All the subjects, V, VI and VII-'35 were young graduate students, one 25 and the other two 24 years of age. Subjects V and VI were slender, subject VII quite stout, almost obese. None of them had had previous experience with high fat diets, but all exhibited higher tolerance than the three preceding subjects. This may be indicated by the following brief table of data borrowed from the succeeding article.

TABLE 8

*Indicated tolerance for fat in basal period on fourth day of all-cream diet*

SUBJECT	KETONEMIA	KETONURIA
	<i>mg./100 cc.</i>	<i>mg./hr.</i>
I	31.9	308
II	50.9	237
IV	55.3	264
V	21.0	38
VI	20.9	71
VII-'35	31.4	95
VII-'36	14.2	25
VIII	36.4	176
IX <sup>1</sup>	25.8	210

<sup>1</sup> Fifth day.

As previously emphasized by Hawley, Johnson and Murlin ('33) there is no very close correlation between the respiratory quotient and ketonemia or ketonuria, but a low figure in both these indices combined with high or 'normal' R.Q.'s denotes high tolerance; high figures for ketosis and low for R.Q.'s, low tolerance.

These subjects, accordingly showed very few low quotients, the lowest (0.666, non-protein 0.64) occurring as a basal period with subject V. Subject VI gave no non-protein R.Q.'s below 0.70 and subject VII-'35 only one (0.69 in the third calorimeter period on June 14th; table 4). The response to the high fat meal, which consisted of 400 gm. (or slightly more) of the heavy cream in all three cases, was an average non-protein R.Q., for the three full-hour calorimeter periods, of 0.74, 0.74 and 0.72. The specific dynamic effect was an increased heat production which averaged for the same three periods, respectively, 15.4, 21.4 and 23.1% above basal. Table 4 illustrates this group of experiments.

The S.D.A. curves for subjects V, VI and VII are given in figures 4, 5 and 6. The biphasic appearance of two of these may be due to the almost uncontrollable somnolence which is produced by the high fat metabolism at about the end of the third hour, or to periodicity in the evacuation of fat from the stomach, or both. One subject certainly slept a few minutes in this middle period and the others were not quite sure but that they had fallen asleep momentarily. The 'anaesthetic effects' of diacetic acid have been established by Peterman ('24) and commented upon from this laboratory elsewhere (Marsh and Murlin, '28). The low point in the middle period is followed by a high point the next, which may be accounted for by a fresh evacuation of the cream to the intestine by the stomach. Subject VII-'35 was certainly a little more restless in the calorimeter than the other two.

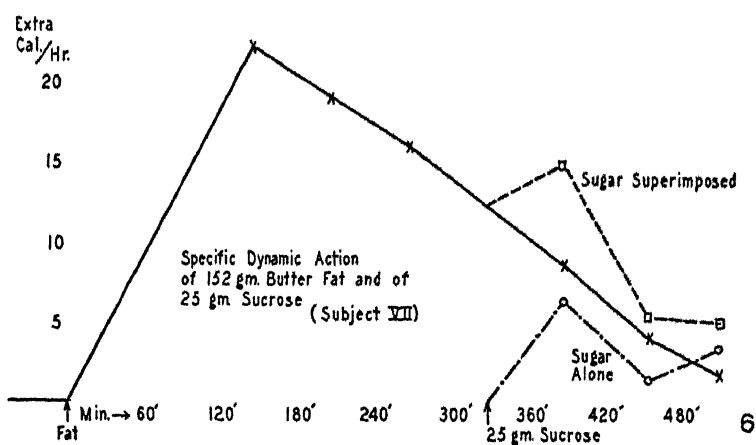
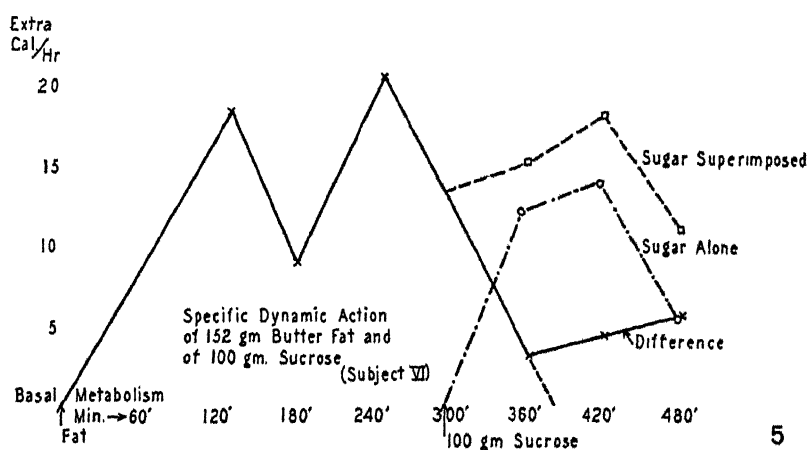
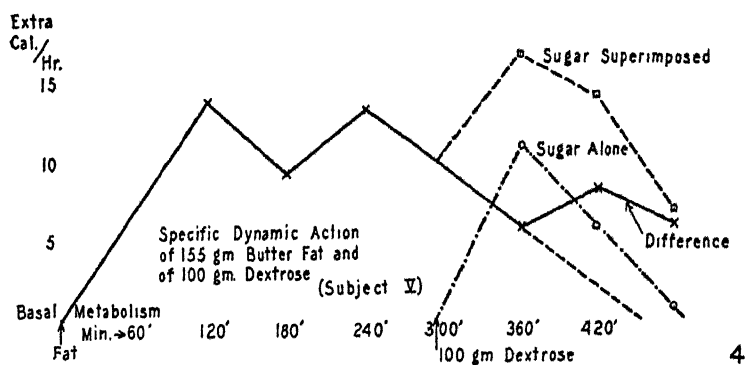
The last three experiments were performed in June 1936. Subject VII was the same individual who bears this number in the 1935 series. The two experiments are distinguished by the year date, thus (VII-'35 and VII-'36). Because he was

TABLE 4

Specific dynamic action of butter fat and of sucrose superimposed (subject VII-195)

DATE 1934 JUNE	TIME <sup>1</sup> AFTER SUGAR <i>minutes</i>	TIME <sup>2</sup> AFTER FAT MEAL <i>minutes</i>	APPLIANCE	RESPIRATORY EXCHANGE						HEAT CAL./HR.		S.D.A. PER CENT OF INDIRECT
				Period	CO <sub>2</sub>	O <sub>2</sub>	R.Q.	N/hr.	NON- PROTEIN R.Q.	Indirect	Direct	
13		<i>minutes</i> Basal	T	9.36-9.46 A.M. 10.40	L./hr. 13.95	L./hr. 17.30	0.81	0.334	0.81	82.6		
				11.47-12.48 P.M.	In calorimeter—no food—nude							
				12.48-1.42 P.M.	12.55	16.32	0.77	0.297	0.76	77.0	81.1	
14		Basal	T	12.48-1.42 P.M.	12.94	16.79	0.77	0.297	0.77	79.4	82.0	
				8.59-9.09 A.M.	13.65	17.47	0.78	0.376	0.78	82.7		
				9.25-9.35 A.M.	13.37	17.55	0.76	0.376	0.75	82.6		
			T	9.45 A.M. 10.00 A.M.	Took 400 gm. 4X cream = 152 gm. fat In calorimeter—false start							
				11.42-12.42 P.M.	16.48	22.46	0.73	0.520	0.72	104.9	101.3	26.8 <sup>4</sup>
				12.42-1.42 P.M.	14.98	19.62	0.76	0.520	0.75	92.3 <sup>1</sup>	101.7 <sup>3</sup>	23.1
			C	1.42-2.44 P.M.	15.29	21.51	0.71	0.520	0.69	98.6 <sup>2</sup>	98.0	19.3
				3.13 P.M.	Took 25 gm. sucrose in 300 cc. water					295.8	301.0	
				4.09-4.19 P.M.	15.34	20.84	0.73	0.454	0.73	97.5		18.0
			T	5.14-5.24 P.M.	15.51	18.31	0.84	0.454	0.85	88.0		6.4
				6.10-6.20 P.M.	15.89	18.09	0.87	0.454	0.89	87.6		6.1
				6.55 P.M.	Took 400 gm. 4X cream + 100 gm. lettuce							
15		Basal	T	3.05 A.M.	Took 25 gm. sucrose in 300 cc. water							
				8.48-8.58 A.M.	12.93	16.77	0.77	0.307	0.77	79.4		
				9.17-9.27 A.M.	12.73	17.18	0.74	0.307	0.73	80.5		
		Basal	T	9.55 A.M.	Took 25 gm. sucrose in 300 cc. water							
				11.04-11.14 A.M.	13.71	18.34	0.74	0.321	0.74	86.2		7.8 <sup>5</sup>
				12.03-12.13 P.M.	13.43	17.14	0.78	0.321	0.78	81.3		1.6
			T	1.01-1.11 P.M.	13.88	17.50	0.79	0.321	0.79	83.2		4.1

<sup>1</sup> Subject slept few minutes.<sup>2</sup> Subject slept few minutes.<sup>4</sup> Using 82.6 Cal./hr. mean of indirect as basal.<sup>5</sup> Using 79.9 Cal./hr. mean of indirect as basal.



Figures 4 to 6

rather uncomfortable in the calorimeter the entire series of respiratory determinations with him in 1936 was carried out by means of the Tissot-Haldane procedure. In the 1935 experiment he had exhibited rather high tolerance for the cream diet; the ketosis was low (table 3) and the R.Q.'s high. In the 1936 experiment, however, he had considerable stomach distress causing hyperpnoea and as a consequence gave two periods with excessively high R.Q.'s after taking 350 gm. of the cream (table 5). The R.Q. for the third period of this test is probably correct, for the symptoms of gastric distress and hyperpnoea had entirely passed off. The dynamic effect of this meal is comparable with that of the corresponding meal in 1935 (table 4, June 14th) obtained with the calorimeter. Two of the periods in each case center at the same intervals after taking food. The sum of the extra heats calculated to 1 hour in each case is 41.4 Cal. for the meal of 152 gm. fat in 1935 and 40.4 Cal. for the meal of 144 gm. fat in 1936—very nearly an exact proportion. Slight discomfort in the calorimeter in the first test was matched by the over breathing in the second. Both give dynamic effects above the average (table 8).

The next day (fifth of high fat) in the 1936 experiment shows several abnormally low R.Q.'s both in the early morning and later morning periods. Two of these occur after 25 gm. glucose had been given, clearly demonstrating that a similar dose of sugar the previous day had not restored the glycogen of the liver sufficiently to permit combustion (see following paper). To the same effect is the logical inference from the low quotients earlier in the day. Also the ketosis by this time had risen considerably. The combined dynamic effect of sugar and fat, however, is larger on this than on the preceding day (table 5).

Subject VIII had the highest ketosis on the fourth day of diet of the last group (table 3). Accordingly, in the basal determination in the calorimeter on June 1st (table 6) he shows one abnormally low R.Q. and one also after taking the meal of 330 gm. 4X cream. The dynamic effect of the meal is low as

TABLE 5  
Specific dynamic action of butter fat and of superimposed sugar (glucose) subject VII-'36

DATE 1936 JUNE	TIME <sup>2</sup> AFTER SUGAR	TIME <sup>2</sup> AFTER FAT	APPLIANCE	RESPIRATORY EXCHANGE				NITROGEN IN URINE	NOR- PROTEIN R.Q.	HEAT CAL./HR. INDIRECT	S.D.A. EXTRA CAL./HR. OVER BASAL	PER CENT OF INDIRECT BASAL
				Period	CO <sub>2</sub>	O <sub>2</sub>	R.Q.					
8 <sup>1</sup>	minutes	minutes										
	Basal	Basal	T	8.31-8.41 A.M.	12.85	17.25	0.75	0.396	0.74	80.85	Average basal	
	Basal	Basal	T	9.15-9.25 A.M.	13.06	17.45	0.75	0.396	0.74	81.87		
				8.36 A.M.	350 gm.	4X cream (144 gm. fat)						
		85	T	10.56-11.06 A.M.	17.48	20.11	0.89	0.483	0.88	97.29	15.93 <sup>4</sup>	19.6
		145	T	11.56-12.06 P.M.	17.87	20.86	0.86	0.483	0.86	100.64	19.28 <sup>4</sup>	23.7
		205	T	12.56-1.06 P.M.	16.43	21.81	0.75	0.483	0.75	102.49	21.13 <sup>5</sup>	24.9
		224		1.20 P.M.	25 gm. glucose in 300 cc. water							
	65	289	T	2.20-2.30 P.M.	15.88	20.35	0.78	0.560	0.77	95.99	14.63	18.0
	125	349	T	3.20-3.30 P.M.	14.51	20.02	0.72	0.560	0.71	93.10	11.74	14.4
9	185	409	T	4.20-4.30 P.M.	13.72	19.10	0.72	0.560	0.70	88.74	7.38	9.1
				6.22 A.M.	350 gm.	4X cream (144 gm. fat)						
		138	T	8.35-8.45 A.M.	14.01	19.73	0.71	0.613	0.69 <sup>3</sup>	89.72	8.36	10.2
		178	T	9.15-9.25 A.M.	15.55	21.29	0.73	0.613	0.71	99.13	17.77	21.8
		195		9.35 A.M.	25 gm. glucose in 300 cc. water							
	69	262	T	10.39-10.49 A.M.	15.34	21.50	0.71	0.600	0.70 <sup>3</sup>	98.66	17.30	21.3
	127	320	T	11.37-11.47 A.M.	15.45	21.63	0.72	0.600	0.70	99.36	18.0	22.1
	185	378	T	12.35-12.45 P.M.	15.79	21.31	0.74	0.600	0.73	99.57	18.21	22.4

<sup>1</sup> Fourth day of all-cream diet.

<sup>2</sup> Calculated to middle of experimental period.

<sup>3</sup> Heat value of oxygen by extrapolation from R.Q. 0.707.

<sup>4</sup> Excessive breathing caused by stomach distress, produced high R.Q.'s.

<sup>5</sup> Breathing normal.

TABLE 6

Specific dynamic action of butter fat and of superimposed sugar (fructose) subject VIII

DATE 1936 JUNE	TIME <sup>2</sup> AFTER SUGAR	TIME <sup>3</sup> AFTER FAT	AP- PLIANCE	RESPIRATORY EXCHANGE					NITROGEN IN URINE	HEAT CAL./HR.		S.D.A. EXTRA CAL./HR. OVER INDIRECT	PER CENT INDIRECT
				Period	CO <sub>2</sub>	O <sub>2</sub>	R.Q.	NON- PROTEIN E.Q.		Indirect	Direct		
1 <sup>1</sup>	minutes	minutes			L./hr.	L./hr.			gm./hr.				
	Basal	Basal	T	8.41-8.51 A.M.	13.26	16.92	0.78	0.78	0.504	79.94			
	Basal	Basal	T	9.06-9.16 A.M.	11.93	15.99	0.75	0.73	0.504	74.69			
	Basal	Basal	C	9.40 A.M.	In calorimeter, nude								
	Basal	Basal	C	11.04-12.09 P.M.	11.77	16.60	0.71	0.69 <sup>2</sup>	0.550	75.2	76.1 <sup>4</sup>	{ Average basal 76.93	
	Basal	Basal	C	12.09-1.09 P.M.	12.30	16.75	0.73	0.72	0.550	77.9	77.9		
				1.40 P.M.	330 gm. 4X cream (132 gm. fat)								
				2.00 P.M.	In calorimeter, nude								
		104	C	2.54-3.54 P.M.	11.62	16.38	0.71	0.69 <sup>2</sup>	0.501	74.5	74.5		
		164	C	3.54-4.54 P.M.	13.44	18.12	0.74	0.73	0.501	84.7	79.8	7.77	10.1
2		224	C	4.54-5.54 P.M.	13.51	17.80	0.76	0.75	0.501	83.5	83.13	6.63	8.6
		275		6.15 P.M.	50 gm. C.P. fructose in 300 cc. water								
	70	345	T	7.20-7.30 P.M.	14.58	18.87	0.77	0.77	0.442	89.13		12.20	15.8
	140	415	T	8.30-8.39 P.M.	16.64	18.59	0.89	0.91	0.442	80.55		13.62	17.7
	198	473	T	9.30-9.37 P.M.	20.02	17.99	1.11	1.16	0.442	92.07		15.14	19.6
				4.00 A.M.	330 gm. 4X cream (132 gm. fat)								
		318		8.44-8.50 A.M.	10.51	18.36	1.06	1.09	0.268	93.32		16.39	21.4
		287	T	9.18 A.M.	50 gm. C.P. fructose in 300 cc. water								
	68	386	T	10.23-10.29 A.M.	20.61	18.46	1.12	1.15	0.283	94.89		17.96	24.4
	129	447	T	11.24-11.30 A.M.	19.40	16.73	1.16	1.20	0.283	86.73		9.80	12.7
	189	507	T	12.23-12.28 P.M.	22.06	17.78	1.25	1.29	0.283	93.77		16.84	21.9

<sup>1</sup> Fourth day of all-cream diet.<sup>2</sup> To middle of experimental period.<sup>3</sup> Heat value of oxygen by extrapolation.<sup>4</sup> Using rectal temperature only for this period.

was observed with subject II (p. 621), and with subject IV (p. 624 in table 2) under similar conditions. The ketosis in subject VIII, however, was not quite so high and the R.Q.'s not so low as with subject IV. The low R.Q.'s while not directly proportional to the ketosis, evidently are related to poor combustion and therefore to the specific dynamic effect of the fat, for subject VIII gave no dynamic effect the first calorimeter hour after the fat meal (really the second hour after the meal) when the R.Q. was lowest, and quite low figures even when the R.Q.'s were normal. Respiration was normal in the calorimeter.

This subject's respiratory center, however, became very sensitive to ketosis later, for in the early morning periods on June 2nd, his respiration rate rose to 30 per minute. The second early morning period (which is shown in table 6) it had fallen to 24 per minute, but the R.Q. is still abnormally high. To some extent a high respiration rate is responsible for the high quotient following the ingestion of C.P. fructose the day before; but the highest quotient accompanied the lowest rate and therefore can scarcely be due to the acidosis alone. Carpenter and Fox ('30) have shown that doses of levulose as small as 5 gm. perceptibly raise the R.Q. and Carpenter and Lee ('33) have shown that the rise in quotient after 25 gm. fructose is not due to the formation of organic acids causing diminished alveolar  $\text{CO}_2$ . Quotients higher than unity so often obtained with this sugar, without any acidosis Carpenter believes, are only to be explained by the formation of fat (see also Deuel, '27).

In the present instance we cannot with confidence explain the high respiratory quotients as due to fat formation because of the known ketosis and the signs of respiratory stimulation.<sup>6</sup> The sugar was given to determine its specific dynamic effect when superimposed on fat, to compare this combined effect with that of other sugars, and also to observe its effect on the ketogenesis (see following paper). With full reservation as to the correct explanation of the high quotients, the results on dynamic action are shown in table 6. The high quotient in

<sup>6</sup> See table 7, following article, for effect on  $\text{CO}_2$ -combining power of the blood.



the early morning period on June 2nd, 4½ hours after fat, must be due in large measure to the ketosis causing a high respiration rate. The dynamic effect of fat at this time, however, calculated on the high non-protein R.Q. with allowance for fat formation is no higher than is seen in many other subjects (table 8). Further comment on the effect of sugar will be found in the following sections.

Subject IX developed ketosis on the high fat rather more slowly than any other subject (table 3). His tolerance for fat from every other observable criterion was good, yet he showed a number of low R.Q.'s by both methods, some of them even after sugar was ingested. Table 7 shows many other interesting details. The specific dynamic action of 123 gm. fat with not over 6% of the calories from other food stuffs is calculated from a curve constructed from only the five periods after fat on June 23rd and 24th. The average coincides with the grand average shown in table 8.

A summary of the specific dynamic action of butter fat in the nine subjects is assembled in table 8. The extra calories per hour are derived from S.D.A. curves, as shown in the representative figures, by dropping perpendiculars from the curve to the base line at the middle point of the successive hours spanned by the curve. The summation of these successive hourly levels is substantially equivalent to the area under the curve. The two experiments on subject I at the end of the table taken from data published previously, are introduced for comparison because there was an actual determination of the heat production at intervals of 1½ hours all through the day up to 9 hours after the meal in one and 10½ hours in the other. These two curves from complete records give final values which are within the same range as the others in the table. The summation of total S.D.A. calories, expressed as a percentage of the fat calories contained in the test meal, shows a range for the subjects able to maintain a perfectly satisfactory state of repose of 3.55 to 6.0 with a mean value of 4.74%. The corresponding value given by Murlin and Lusk for dogs ('15) is 4.1%. Subjects II and VII-'35 are excluded

TABLE 7  
Specific dynamic action of butter fat and of superimposed sugar (sucrose) subject IX

DATE 1936 JUNE	TIMES <sup>2</sup> AFTER SUGAR	TIMES <sup>3</sup> AFTER FAT	AP- PLANCE	RESPIRATORY EXCHANGE				NITROGEN IN URINE	NON- PROTEIN R.Q.	HEAT CAL./HR.		S.D.A.:	
				Period	CO <sub>2</sub>	O <sub>2</sub>	R.Q.			Indirect	Direct	Extra Cal./hr. over indirect	Per cent of indirect
22 <sup>1</sup>	minutes	minutes			L./hr.	L./hr.		gm./hr.					
	Basal	Basal	T	8.25-8.35 A.M.	11.93	16.25	0.73	0.773	0.70	75.1			
	Basal	Basal	T	9.02-9.12 A.M.	12.31	16.33	0.75	0.773	0.72	75.7			
	Basal	Basal	C	9.50 A.M. In calorimeter, nude									
23			C	11.04-12.10 P.M.	11.62	15.73	0.74	0.756	0.72	74.8	75.7	Average	
			C	12.10-1.04 P.M.	12.35	16.51	0.75	0.756	0.72	76.6	73.6	basal	
				1.22 P.M. 300 gm. 4X cream (123 gm. fat)								75.19	
		245	T	5.22-5.32 P.M.	12.89	18.37	0.70	0.721	0.67 <sup>2</sup>	81.77		6.58	8.7
		305	T	6.22-6.32 P.M.	13.56	18.31	0.74	0.721	0.72	85.08		9.89	12.0
			T	8.32-8.42 A.M.	12.19	16.45	0.74	0.634	0.72	76.43			
			T	9.12-9.22 A.M.	12.18	16.41	0.74	0.634	0.72	76.26			
				9.47 A.M. 300 gm. 4X cream (123 gm. fat)								Average	
				10.45 A.M. In calorimeter, nude								76.34	
		142	C	11.33-12.42 P.M.	13.74	19.77	0.70	0.631	0.67 <sup>2</sup>	87.85	.... <sup>4</sup>	11.55	15.2
		205	C	12.42-1.45 P.M.	13.75	19.67	0.70	0.631	0.67 <sup>2</sup>	87.84	85.06	11.60	15.3
		262	C	1.45-2.33 P.M.	14.47	19.67	0.74	0.631	0.72	91.58	90.12	15.24	20.0
24		303		2.50 P.M. 50 gm. sucrose in 300 cc. water									
	66	369	T	3.51-4.01 P.M.	12.99	18.58	0.70	0.595	0.67 <sup>2</sup>	83.09		6.75	8.8
	127	430	T	4.52-5.02 P.M.	13.27	18.19	0.73	0.595	0.71	84.52		8.18	10.7
	187	490	T	5.52-6.02 P.M.	12.02	16.37	0.73	0.595	0.71	76.06		0.0	0.0
				4.55 A.M. 300 gm. 4X cream (123 gm. fat)									
		247	T	8.57-9.07 A.M.	13.87	18.25	0.76	0.417	0.75	85.76		9.42	12.2
		292	T	9.42-9.52 A.M.	13.64	18.63	0.73	0.417	0.72	86.99		10.65	14.0
		306		10.01 A.M. 50 gm. sucrose in 300 cc. water									
	67	373	T	11.03-11.13 A.M.	14.72	19.47	0.75	0.426	0.74	91.57		15.23	19.0
	125	431	T	12.01-12.11 P.M.	14.39	19.37	0.74	0.426	0.73	90.67		14.33	20.0
	186	492	T	1.02-1.12 P.M.	11.32	15.80	0.71	0.426	0.70	73.57		0.0	0.0

<sup>1</sup> Fourth day of all-cream diet.

<sup>2</sup> Calculated to middle of experimental period.

<sup>3</sup> Heat calculated by extrapolation to this R.Q.

<sup>4</sup> Obvious large error on direct heat.

TABLE 8  
*Specific dynamic action of butter fat expressed as a percentage of the calories fed*

SUBJECT	AGE	WEIGHT kg.	EXTRA CALORIES PER HOUR										NUMBER HOURS AVERAGE	EXTRA CALORIES SUM- MATION	FAT CALORIES IN FOOD	TOTAL S.D.A.	AVERAGE EXTRA CAL./HR.
			1	2	3	4	5	6	7	8	9	10					
I	60	94	2.5	6.8	11.4	12.5	9.7	6.8	3.8	1.0			7.8	54.5	1534	3.55	7.0
II	31	75	4.5	14.3	12.3	10.2	8.0	6.0	3.8	1.7			8.2	60.8	819	(7.42) <sup>2</sup>	7.4
III	45	68	2.2	6.6	12.7	16.7	16.6	11.6	6.7	1.5			7.8	74.6	1368	5.45	9.6
IV	23	88	1.7	5.1	9.2	7.2	6.0	2.5					5.8	31.7	855	3.70	5.4
V	25	73	3.5	10.7	11.2	11.2	11.3	7.7	3.3	0.0			7.5	58.9	1395	4.22	7.8
VI	24	70	4.0	12.9	15.2	15.0	18.0	8.5	0.0				6.4	73.6	1368	5.38	11.5
VII-'35	24	92	4.5	13.6	22.1	19.0	15.8	12.2	8.5	4.3	1.7		8.5	101.7	1368	(7.43) <sup>2</sup>	12.0
VII-'36	25	92	2.5	7.2	11.8	16.5	17.0	12.7	8.5	4.2			8.0	80.4	1339	6.0	10.0
VIII	25	71	1.7	5.1	8.6	12.0	16.0	12.7	7.7	2.5			8.0	66.3	1227	5.4	8.3
IX	27	73	2.0	6.1	10.2	14.2	15.0	6.0	0				6.0	53.5	1144	4.7	8.9
I'	56	93	5.2	15.8	16.7	16.2	14.1	15.0	13.6	9.7	7.3	2.0	10.0	115.6	2250	5.13	11.5
I'	56	93	4.3	13.3	14.3	13.7	11.4	11.1	8.0	2.1	5.0	2.8	10.0	86.0	2250	3.8	8.6
											Average		7.0	71.4	2024	4.74	9.0

<sup>1</sup> Taken from paper by Hawley, Johnson and Murlin ('33).

<sup>2</sup> Subject somewhat restless; not used in average.

from this average because the former was considerably upset by the diet on one of his experiments and the latter was somewhat 'fidgety' in the calorimeter. The lowest dynamic effects by this index were shown by the oldest subject and by the youngest, who as it happens was quite corpulent and not at all inclined to muscular exercise. Subject VII was equally corpulent but was at the same time quite active muscularly. Aside from the subjects II and VII-'35 the next highest score was made by subject III, a man who lives by manual labor, and subject VI, who was well-developed muscularly and kept in training by playing squash frequently.

#### THE S.D.A. OF SUPERIMPOSED SUGAR

This is well shown by the curves in figures 1, 3, 4, 5 and 6. There are two types of response: 1) fairly complete summation; and 2) greater effect when sugar is superimposed than when taken alone. The first is illustrated by figures 3 and 6 and is recognized by the fact that the curve for fat (continuous line) falls at a uniform rate, where, toward the end, the values for sugar alone are subtracted from the curve obtained by superimposition. The second type is illustrated by all the other curves showing effects of sugar (figs. 1, 4 and 5). There were no separate determinations of the dynamic effect of sugar alone for the last three subjects. The first type is the one which would be expected if there is no interference with absorption of either food when mixed in the alimentary tract. Murlin and Lusk ('15) obtained nearly complete summation of the separate dynamic effects of fat and glucose and of these two with glycocoll in the dog. Type 2 is the more interesting because it seems to denote a more favorable utilization of the fat when sugar is superimposed upon it. As shown most strikingly in figure 4, the dynamic effect of fat and sugar is so much greater that when the effect of sugar alone is subtracted, there remains quite a hump of extra dynamic effect on the fat curve (marked 'difference'). This particular curve is selected for illustration because the subject (V) was probably the most completely reposed subject

who has ever been in the calorimeter, and he was equally so on the cot for Tissot-Haldane determinations. A possible explanation of this phenomenon that can be offered is along the line of the observations by Woods and Merrill ('00) that protein of bread shows a higher coefficient of digestibility when eaten with milk than when eaten alone. Hosoi, Alvarez and Mann ('28) found the same applied to egg white and milk protein. There is ordinarily no interference of fat in the diet with the absorption of other foods (Langworthy and Holmes, '15). While in these observations no systematic controls have been made by ingestion of water alone at a corresponding period after ingestion of fat, it has been observed several times that ingestion of water between periods in order to relieve gastric distress, did not seem to affect the dynamic curve. At all events it would be very surprising if absorption or metabolism of fat would be accelerated to the extent of 8 Cal. per hour by a drink of 300 cc. of water. A still better explanation would seem to be the aid to better oxidation of fat which sugar is known to provide (Shaffer, '21 a, b, c). The next paper will give the data regarding concurrent reduction of ketone bodies. This is the first observation on record, so far as can be discovered, that such a reduction is accompanied by increased heat production.

#### S.D.A. OF SUGAR WITHOUT EVIDENCE OF COMBUSTION

The object of giving sugar was to test its combustibility at two different intervals after the fat meal. This test is connected with the possible formation of glycogen from fat as developed in an earlier paper (Hawley, Johnson and Murlin, '33). The work is not yet complete and will be reported at a later date.

The two intervals chosen for most of the experiments were 3 to 5 hours after the fat meal, called hereafter 'early' and 11 to 15 hours after, called hereafter 'late,' i.e., at a time when the dynamic action of fat may be supposed to have passed off (fig. 2). With the last three subjects the intervals after fat for giving the sugar were approximately the same. This

was accomplished by giving an extra meal of fat very early in the morning (tables 5, 6 and 7) and following with sugar without an intervening basal. The same amount of sugar was given at the two feedings. In the case of subject VII-'35 (table 4) two doses of sucrose were, for a special purpose, given in the morning, one at 3.05 A.M. and the other at 9.55. Basals were begun at 5 hours and 40 minutes after the first sugar. In spite of these two doses the R.Q.'s following the second showed only a slight rise, proving more conclusively than in any other experiment that the sugar was used chiefly to replenish glycogen. The intervals after sugar ingestion at which the respiratory metabolism was determined by the Tissot-Haldane method were purposely kept about the same (see tables 1 to 7, except 3) for the afternoon, following fat in the morning, and for the early morning experiments.

The work of Mason ('25, '26) and of Deuel ('27) is confirmed by this study in proving that the S.D.A. of carbohydrate cannot be explained by the plethora theory of Lusk. With characteristic frankness, Lusk ('31 a, b) admitted this in his last reviews of the subject. The dynamic rise caused by sugar without any increased combustion of sugar (indeed a fall of a few carbohydrate Calories per hour) is most clearly shown for subject VII-'36 in table 5, and for subject VI in figure 5; but it is also shown for subject I in figure 1, and with only a very slightly increased combustion (only 2 carbohydrate Cal. per hour) in figure 4. Even with subject IV (table 2) who shows an average rise of non-protein R.Q. of 0.08 (from 0.64 to 0.72) the dynamic effect when sugar was fed 'early' after fat produced only 4.3 carbohydrate Cal. per hour, although the total dynamic rise due to sugar was 6.4 Cal. per hour. The next day when fat was not being absorbed the same ingestion of sugar produced a similar average rise of non-protein R.Q. (0.706 to 0.801) and resulted in the combustion of much more sugar—an increase of 23.3 Cal. per hour. With subjects II and VII-'35 the dynamic effect of the sugar was identical whether fed 'early' or 'late' (figs. 3 and 6). The increased combustion of sugar which entered into this effect,

however, was very different in the two subjects. Subject II increased his carbohydrate Calories 30.6 and 33.0 per hour, respectively, in the two conditions, the only one to come so near an equality. Subject VII-'35 experienced an increase in R.Q. from sugar over four times as great in the 'early' state as in the 'late,' notwithstanding an extra 25 gm. sucrose in the very early morning of the 'late' experiment.

The last three subjects afford additional evidence. The second dose of sugar with these subjects followed last fat at the same interval as the first, and yet it sustained a higher combined dynamic effect with no greater increase of non-protein respiratory quotients (tables 5, 6 and 7). Making allowance for better tolerance of fat with developing adaptation, there is no reason to suppose the sugar was not equally well absorbed. Clearly, therefore, there is no proportionality between the specific dynamic effect of sugar and the amount of sugar burned.

#### SUMMARY AND CONCLUSIONS

1. The specific dynamic action of butter fat, eaten as heavy cream, was studied in nine human subjects by means of the new semi-automatic respiration calorimeter, supplemented by the Tissot-Haldane method. Amounts of fat contained in the test meal varied from 80 to 171 gm. S.D.A. experiments occurred on the fourth or fifth day of an all-cream diet.

2. For non-protein respiratory quotients less than 0.707, heat values for oxygen below the Zuntz-Shumburg table were found by the formula  $\frac{4.686 \times \text{low R.Q.}}{0.707}$ . The method assumes that all of the non-protein  $\text{CO}_2$  under these conditions comes from combustion of fat and that all of the extra oxygen is used for some other purpose than immediate combustion. Direct and indirect calorimetry agreed remarkably well, thus tending to confirm the assumption.

3. Plausible S.D.A. curves showing the entire course of extra heat production over basal were possible for all subjects but one. The average S.D.A. expressed as a percentage of the fat calories fed was 4.74. Not more than 6% of the calory

content was from other foodstuffs. The figures agree fairly well with those of Murlin and Lusk ('15) on the dog.

4. There is no relation of S.D.A. to age, for lowest effects were obtained from the youngest and oldest subjects. It is, however, related to tolerance for fat, meaning not only capacity to digest but also capacity to metabolize completely.

5. With six subjects sugar (sucrose or glucose C.P.<sup>\*</sup>) was superimposed on the high fat at an 'early' interval (3 to 5 hours) after the fat meal was taken and the same amount again at a 'late' interval (11 to 15 hours), for the purpose of testing its combustibility. The dynamic response to the sugar was of two types, a) complete summation to the fat metabolism, and b) an increase greater than that due to the same amount of sugar fed alone; the difference is reckoned as fat metabolism. The extra heat may come in part from oxidation of ketone bodies induced by the sugar (see following paper). With three subjects the sugar, glucose, fructose and sucrose, respectively, was given twice at equal intervals after last fat and the combustibility and dynamic effects compared.

6. There is no doubt from these experiments that a strong dynamic effect from sugar may be obtained without any evidence of its own combustion; where such evidence (higher R.Q.) was obtained there was no proportionality to the dynamic effect.

7. The S.D.A. of fat does not appear to be due entirely to plethora in the sense of Lusk, for a) it does not run parallel to blood fat, and b) sugar superimposed causes increased combustion of fat.

<sup>\*</sup> Supplied by Corn Industries Research Foundation.



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# THE RATE OF KETOGENESIS IN HUMAN SUBJECTS ON HIGH FAT DIETS, AS INFLUENCED BY DIFFERENT SUGARS

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## INTRODUCTION

The important work of Shaffer ('21, '22) and of Woodyatt ('21) indicated a close quantitative relationship of ketogenic to antiketogenic factors in the mixture of foodstuffs being oxidized in the body at the threshold of ketonuria. It is as true now, as it was 15 years ago, that the practical management of ketosis is best guided by these quantitative relations. The threshold of ketonuria has been found to occur at or near the K:A ratio of Shaffer or the FA:G ratio of Woodyatt by several different observers (Hubbard and Wright, '22; McClellan, Spencer, Falk and DuBois, '28; McClellan and DuBois, '30). Beyond the threshold, however, important exceptions in yield of ketones in the urine, as compared with the theoretical yield according to Shaffer, have been encountered, especially in obese and other fat-adapted subjects (McClellan, Spencer, Falk and DuBois, '28; Hawley, Johnson and Murlin, '33); in persons living on an exclusive meat diet (McClellan and DuBois, '30), and very recently in depancreatized dogs whose metabolism was increased by exercise and by dinitrophenol (Barker, '36). A further examination of the question of ketone yields in comparison with combustion of carbohydrate, from the standpoint of changes occurring over short periods of time, and employing a new manner of expressing the ketogenesis, would seem to be in order.

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In an earlier paper from this laboratory, it was shown that no relation seemed to exist between the level of the R.Q. in persons living on an all cream diet (Hawley, Johnson and Murlin, '33) and the degree of ketosis as measured either by ketonuria or ketonemia. In another communication (Clarke and Murlin, '36) ketolytic effects without evidence of combustion of carbohydrate have been noted in depancreatized dogs and in still another (Murlin and Manly, '36) where the effect of different sugars on ketosis in 4-hour periods, was studied, the greatest reduction was not obtained by the most combustible sugar. In this latter paper a method suggested by Manly of expressing the effect of sugar by means of the change in 'ketone substance production' was adopted. This term is synonymous with the term 'rate of ketogenesis' as used in the present paper. It takes into account both the ketonemia, or level of ketone bodies in the blood, and the ketonuria, or the rate of ketone body excretion in the urine. The amount of ketone bodies excreted, expressed in mg./hr. is increased or decreased, as the case may be, by the change in ketone level multiplied by blood volume, expressed again in mg./hr., according to whether there has been an accumulation of ketones in the circulation or a loss therefrom. The literature bearing on the effects of different sugars on ketosis has been sufficiently reviewed in the two papers just cited.

That aspect of the quantitative relationship of ketolytic to ketogenic factors which has interested us especially in the present study on human subjects, is the relation of carbohydrate combustion to the reduction of ketogenesis. If the body depends upon a stoichiometric combustion of sugar with fatty acid to prevent ketosis, the cure of an already established ketogenesis by feeding sugar should show some evidence of combustion of the sugar.

The relation of ketogenesis to glycogen formation also is important. Concerning this question, it may be pointed out that much new evidence points to the liver as the principal site of ketogenesis (Burn and Ling, '28; Cannavó, '31; Leites and Odinov, '35; Beazell, '35; Edson, '35 a and b; Mirsky,

'36 a and b) as well as the site of other transformations of fat (Gemmell and Holmes, '35; Haarmann, '35); it might be expected, therefore, that the effects of sugar could be explained principally in terms of what happens in the liver. Since this cannot be determined directly in the human subject, one is compelled to rely upon the respiratory metabolism and changes in the blood and urine, which are closely related to glycogen supply and combustion of carbohydrate. It is generally agreed that if the combustion of a dose of sugar follows promptly after its administration, the glycogen level of the liver is relatively high, or at least that the liver is not being hindered in its formation of glycogen. This is one inference which may fairly be drawn from the alteration of tolerance curves (du Vigneaud and Karr, '25) and of the R.Q.'s (Dann and Chambers, '30) with repeated doses of sugar administered to fasting animals. The bearing of the experiments to be described upon the glycogen level as measured by the combustibility of sugars will be discussed.

The paper immediately preceding this one contains the effects on heat production of superimposing sugar at two different intervals after a meal of fat. The primary object of thus spacing the meals of sugar was to test its relative combustibility at two stages of the fat metabolism, one (early) 3 to 5 hours, after fat when ketosis was highest and the other (late) 11 to 15 hours after, when ketosis was definitely on the decline and the specific dynamic action of fat entirely had disappeared. There were considerations with reference to the glycogen storage in the liver also which led to this manner of feeding, but this phase of the investigation has not been completed and will be reported upon at a later date.

Would the effect upon ketogenesis of the two feedings of sugar be proportional to or in any other way ascribable to the increased combustion of sugar? If not, it would appear that a revision of current ideas regarding the prevention of ketosis is necessary. The results from the described spacing of sugar meals have been controlled also by feeding equal doses at equal intervals after fat.

## METHOD

Along with the calorimetric work for determination of the S.D.A. of fat (see preceding paper) urine was collected in the appropriate intervals for calculation of the non-protein R.Q. The same collections were used for determination of the ketone bodies. In all but the last three subjects the Hubbard ('21) method was used following oxidation of interfering substances with sodium peroxide and oxidation of  $\beta$ -hydroxybutyric acid to acetone by potassium dichromate. In the last three subjects Van Slyke's ('17) method was used for the total ketones.

Blood samples were taken also, usually just before the urine was collected so that blood intervals and urine periods practically were identical. In six subjects the Shaffer-Hubbard distillations as slightly modified by Behre and Benedict ('26) was used for the ketone bodies of the blood. Instead of the colorimetric determination, however, the Hubbard idometric determination was used. With the last three subjects the Van Slyke and Fitz ('19) method was employed for the blood filtrates. The recovery of pure  $\beta$ -hydroxybutyric acid added to urine and to blood filtrates in both methods averaged close to 75%. Correction has been made on this basis. With the last three subjects also the  $\text{CO}_2$  combining power of blood plasma, according to the method of Van Slyke and Neill ('24) was determined.

Only eight of the nine different subjects used in the preceding study furnished bloods for the present study of ketogenesis, but subject VII was used twice. Six of them took the supplementary feeding of sugar from 3½ to 5 hours after the test meal of cream and again the following morning, 11 to 15 hours after further ingestion of cream in the evening. The last three subjects took equal doses of the same sugar at the same intervals after feeding fat both days. Four subjects took sucrose, four C.P. corn glucose and one C.P. fructose (Pfanstiehl).

The procedure with reference to administration of fat meals, the calorimetric determinations and respiratory metabolism,

to which the subject of this paper is so intimately related, have been described fully in the preceding paper. It is necessary to refer the reader to this paper for further details.

## RESULTS

Tables 1 to 4 illustrate the fundamental data. Each table concerns one subject, whose roman number is the same as in the preceding paper. Because of limitation of space it is not possible to publish all of the tables. Both in blood and urine acetone and aceto-acetic acid, where this fraction was determined separately, is expressed as acetone,  $\beta$ -hydroxybutyric acid as such and total acetone after conversion of  $\beta$ -hydroxybutyric to acetone. With the Van Slyke and the Van Slyke and Fitz methods only total acetone is reported.

The average non-protein R.Q.'s for the period represented by the urine collection also are given. Blood sugar for subjects I, II, IV and VIII were taken only in relation to the sugar ingestion, but for the remaining five experiments for all blood collections.

The usual increase in ketonemia on the all-cream diet may be noted in tables 1 and 2 from the rise in total acetone from the basal period of the third day to that of the fourth day. Only one exception occurred (subject V), and there is no known explanation. The increase occurs in the  $\beta$ -hydroxybutyric acid in four of the six subjects<sup>2</sup> and in the combined acetone and aceto-acetic in five out of six. With subject V the latter increase is very small. The average increase in total acetone for the six subjects is 8.8 mg.%, which is 33% of the earlier level.

The increase in ketonuria (total acetone) in the basal urine from the third to the fourth day of the same subjects is much greater in absolute figures, but not much greater percentage-wise; namely 141 mg. per hour, which is 41% of the original level (third day).

A single meal of cream given soon after the basal blood on the fourth day of the diet (fifth day for subject IX) and

<sup>2</sup> In table 4 only total acetones are reported.

TABLE 1  
*Effect of glucose on ketosis and ketonuria (subject IV)*

DATE 1934	HOUR	IN BLOOD, MILLIGRAMS PER 100 CC.				AVERAGE NON- PROTEIN R.Q.	IN URINE, MILLIGRAMS PER HOUR			
		Acetone and diacetic as acetone	$\beta$ -oxybutyric acid	Total as acetone	Sugar		Period	Acetone and diacetic as acetone	$\beta$ -oxybutyric acid	Total as acetone
January 30 <sup>1</sup>	9.29 A.M. (basal)	15.8	32.3	33.8		0.67	8.04-9.24 (basal)	8.3	14.5	16.4
	12.23 P.M.	In calorimeter 2 hours—no food				0.7	9.24-12.04	22.7	33.3	41.3
	6.30 P.M.	36.4	43.4	60.6						
		Heavy meal cream + lettuce								
31	9.41 (basal)	25.9	52.7	55.3		0.68	8.11-9.24 (basal)	61.5	364.3	264.8
	10.05	250 gm. 4X cream (95 gm. fat)—in calorimeter 3 hours				0.64	9.24-1.04	88.3	569.8	421.3
	1.00 P.M.	29.0	65.6	65.6	56					
	1.30 P.M.	100 gm. glucose in 300 cc. water				0.72	1.04-4.11	111.0	504.0	392.0
	4.08 P.M.	29.0	43.4	53.2	104					
February 1	6.30 P.M.	250 gm. 4X cream only								
	9.25 (basal)	25.9	80.4	70.8	80	0.71	8.05-9.28 (basal)	13.8	22.2	26.2
	9.45 A.M.	100 gm. glucose in 300 cc. water				0.80	9.28-12.28	9.7	23.9	23.0
	12.27 P.M.	23.6	32.3	41.0	85					

<sup>1</sup>Third day of all-cream diet.



TABLE 2  
*Effect of sucrose on ketosis and ketonuria (subject VI)*

DATE MAY 1935	HOUR	IN BLOOD, MILLIGRAMS PER 100 CC.				AVERAGE NON- PROTEIN R.Q.	IN URINE, MILLIGRAMS PER HOUR			
		Acetone and diacetic as acetone	$\beta$ -oxybutyric acid	Total as acetone	Sugar		Period	Acetone and diacetic as acetone	$\beta$ -oxybutyric acid	Total as acetone
28 <sup>1</sup>	9.40 A.M. (basal)	9.2	16.3	18.3	109	0.73	7.17- 9.33 A.M. (basal)	27.5	52.3	56.5
		In calorimeter 3 hours—no food—no R.Q.'s								
	2.00 P.M.	8.3	16.6	17.6	100		9.33- 2.04 P.M.	28.4	22.1	30.7
	3.00 P.M.	350 gm. 4X cream + lettuce. 6.30 250 gm. 4X cream + lettuce								
29	9.48 A.M. (basal)	11.6	16.6	20.9	120	0.73	7.35- 9.33 A.M. (basal)	26.9	79.6	71.3
		400 gm. 4X cream (152 gm. fat) + lettuce. In calorimeter 4 hours								
	10.10 A.M.	11.1	18.6	21.5	120	0.75	10.20- 3.03 P.M.	57.3	147.0	139.0
	3.06 P.M.	100 gm. sucrose + 300 cc. water								
30	3.10 P.M.	4.6	9.4	9.8	215	0.71	3.03- 6.20 P.M. <sup>2</sup>	41.0	111.8	103.0
	6.35 P.M.	200 gm. 4X cream + lettuce								
	7.00 P.M.	4.3	8.9	9.3	126	0.75	7.49- 9.33 A.M. (basal)	11.0	12.8	18.0
	9.30 A.M. (basal)	100 gm. sucrose + 300 cc. water								
	9.35 A.M.	2.9	7.4	7.0	137	0.86	9.33-12.44 P.M. <sup>3</sup>	6.0	32.8	24.0
	12.50 P.M.									

<sup>1</sup> Third day of all-cream diet.<sup>2</sup> 2.45 gm. glucose in urine.<sup>3</sup> 2.10 gm. glucose in urine.

varying from 240 to 450 gm. raised the ketonemia within an average period of 4.1 hours about 22%; the average increased excretion in milligrams per hour in the corresponding period is 29%. It is evident that neither the rise in blood ketones nor the ketones in the urine alone can express the full effect of the fat meal. Hence it is important to find a better method of expressing the effect not only of a fat meal but also of ketolytic substances to offset the ketogenic. But before preceeding to this matter the alteration in levels of blood ketones

TABLE 3  
*Effect of fructose on ketosis and ketonuria (subject VIII)*

DATE JUNE 1936	HOUR	IN BLOOD, MILLIGRAMS/ 100 CC.		CO <sub>2</sub> COMBINING POWER	AVERAGE NON-PROTEIN R.Q.	IN URINE, MILLIGRAMS PER HOUR			TOTAL KETOSIS	KETOGENESIS
		Total acetone	Sugar			Hour	Nitro- gen	Total acetone		
1 <sup>1</sup>	9.35 A.M.	36.4		vol. % 43.5	0.75	9.37	504	176	2340	
		In calorimeter, basal, nude								
	1.30 P.M.	37.0		44.5	0.70	1.20	550	302	2400	312.1
	1.40 P.M.	330 gm. 4X cream (132 gm. fat)								
	2.00 P.M.	In calorimeter, nude								
	6.14 P.M.	42.0	67	39.0	0.72	6.02	501	422	2720	490.0
	6.15 P.M.	50 gm. fructose in 300 cc. water								
	9.55 P.M.	35.4	89	38.5	0.95	9.58	442	205	2290	93
2	9.00 A.M.	330 gm. 4X cream (132 gm. fat)								
	9.10 A.M.	41.0	100	38.2	1.02	9.02	268	151	2640	
	9.18	50 gm. fructose in 300 cc. water								
	12.45	32.0	85	44.0	1.21	12.43	283	101	2090	-47

<sup>1</sup> Third day of all-cream diet.

and of their excretion produced by sucrose and glucose may be discussed.

The first or 'early' administration of sucrose came at an interval of 3.5 hours approximately after fat for the first three experiments, at 5 hours approximately for the second three, the difference being explained by the longer calorimetric period in the second group, and 4½ hours for the last three. The next blood sample was taken at 3 hours approximately (average 3.3 hours) from the time of drinking the sugar solution. The average effect of the sugar on blood ketones (total

TABLE 4  
Effect of sucrose on ketosis and ketogenesis (subject IX)

DATE 1936 JUNE	HOUR	IN BLOOD, MILLI- GRAMS/100 CC.		CO <sub>2</sub> COMBIN- ING POWER	AVERAGE NON- PROTEIN E.Q.	HOUR	IN URINE, MILLIGRAMS PER HOUR		TOTAL KETOSIS	KETO- GENESIS
		Ketone bodies as acetone	Sugar				Nitrogen	Ketone substance as acetone		
21 <sup>1</sup>	9.32 A.M.	8.7	73	vol. % 52.8	0.71	9.35 A.M.	845	32	588	
	9.42 A.M.	300 gm. 4X cream (123 gm. fat)								
	12.57 P.M.	14.2	66	54.5	0.72	1.00 P.M.	860	80	955	186
	9.20 A.M.	9.7	67	58.0	0.71	9.22 A.M.	773	25	654	
22		In calorimeter 3 hours basal								
	1.15 P.M.	10.6	62	.... <sup>2</sup>	0.72	1.16 P.M.	756	52	717	69
	1.22	300 gm. 4X cream (123 gm. fat)								
	6.35 P.M.	18.3	58	45.0	0.70	6.36 P.M.	721	63	1240	232
23	9.29 A.M.	25.8	70	56.0	0.72	9.30 A.M.	634	210	1745	....
	9.47 A.M.	300 gm. 4X cream (123 gm. fat)								
		In calorimeter 4 hours								
	2.46 P.M.	34.5	64	50.0	0.69	2.48 P.M.	631	495	2330	637
24	2.50 P.M.	50 gm. sucrose in 300 cc. water								
	6.05	12.9	117	56.0	0.70	6.06 P.M.	595	249	873	— 192
	4.55 A.M.	300 gm. 4X cream (123 gm. fat)								
	9.57 A.M.	36.1	76	58.0	0.73	10.00 A.M.	417	347	2440	
	10.01 A.M.	50 gm. sucrose in 300 cc. water								
	1.24 P.M.	10.3	91	63.0	0.70	1.25 P.M.	426	144	697	— 383

<sup>1</sup> Third day of all-cream diet.<sup>2</sup> Sample lost.

as acetone) was a reduction of 8.3 mg. per 100 cc. The range is quite wide; namely, from an elevation of 4.9 mg.% after 50 gm. glucose, to a fall of 21.6 mg.% after 100 gm. sucrose (table 5). The average percentage reduction with sucrose was 38; with glucose 11. Percentage reduction, however, is not a satisfactory basis of comparison, for the reason that the group who took glucose happened to contain two of the subjects with lesser tolerance (see preceding paper), while the group taking sucrose contained only one such individual.

TABLE 5  
*Comparative effects of sugars on ketonemia*

SUBJECT NO.	AMOUNT	SUGAR	ORIGINAL LEVEL	Change in 3 hours
First dose				
VII-'35	25	Sucrose	33.3	— 6.7
VII-'36	25	Glucose	19.3	+ 1.9
IX	50	Sucrose	34.5	— 21.6
I	50	Glucose	41.4	+ 4.9
VIII	50	Fructose	42.0	— 6.6
IV	100	Glucose	65.6	— 12.4
II	100	Sucrose	48.4	— 6.8
V	100	Glucose	33.0	— 16.0
VI	100	Sucrose	21.5	— 11.7
Average		Sucrose	34.4	— 10.7
Average		Glucose	39.8	— 5.4
Second dose (18 hours later)				
VII-'36	25	Glucose	33.2	— 4.9
VIII	50	Fructose	41.0	— 9.0
IX	50	Sucrose	36.1	— 25.8

The original level of the ketones therefore, when the glucose began to exert its effect, was higher, resulting in lower percentage reductions. Average absolute reduction in milligram per cent is patently the fairer method of comparison. On this basis the reduction of total acetone was 10.7 mg% for sucrose and 5.4 mg.% for glucose. There is no close relationship apparent between size of dose and the effect. Twenty-five grams sucrose in one individual produced as great an effect as

100 gm. in another, and 50 gm. produced a greater effect (subject IX). Fifty grams glucose in one individual produced more than twice as great a rise as 25 gm. in another, while 100 gm. produced approximately the same fall in two others. Wierzuchowski ('25) noted that in phlorhizinized dogs the original level of ketosis seemed to determine the extent of the fall produced by any given dose of glucose (the higher the level the greater the fall) and this is borne out by experiments reported from this laboratory (Murlin and Manly, '36). But the present data on normal humans do not exhibit this phenomenon. The comparisons except in one instance are made between different individuals. It seems impossible to make a comparison of two different doses, whether of the same or different sugars on the same individual, without having a hang-over effect of the first dose on the second unless a very considerable period of time has elapsed between the two tests.

The effect of a second dose of the same sugar on the same individual repeated at the same interval after last fat as the first, so as to secure if possible the same initial level, of ketonemia, is exhibited in the lower part of table 5. The second dose followed the first at an average interval of 18 hours (20, 15 and 19). Without exception the second dose produced a greater effect than the first and in two instances the initial levels were very nearly the same. However, the differences are not great, indicating that if the effect of fat had been permitted to hold sway in those subjects for a few hours longer, the hang-over effect would have disappeared.

It is quite clear from the data presented that sucrose is considerably more effective in reducing ketonemia in 3 hours than is glucose.

#### EFFECT ON KETONURIA

When we assemble the effects on different subjects side by side, as in table 6, it is seen again that there is no proportionality between size of dose and extent of the effect. Twenty-five grams sucrose raised the excretion rate slightly while 50 gm. lowered it considerably and 100 gm. reduced it less.

Twenty-five grams glucose increased the excretion rate approximately one-half as much as 50 gm., while 100 gm. reduced it a little in one case and raised it a little in the other. A high initial level of excretion seems to make the rate more susceptible of reduction than a lower level.

TABLE 6  
*Comparative effects of sugar on ketonuria*

SUBJECT NO.	AMOUNT	SUGAR	KETONURIA, MILLIGRAMS PER HOUR	
			Initial level	CHANGE IN 3 HOURS
First dose				
VII-'35	25 gm.	Sucrose	120	+ 20
VII-'36	25	Glucose	56	+ 141
IX	50	Sucrose	495	— 246
I	50	Glucose	159	+ 301
VIII	50	Fructose	422	— 217
II	100	Sucrose	273	— 90
IV	100	Glucose	421	— 29
VI	100	Sucrose	139	— 36
V	100	Glucose	224	+ 12
Second dose (18 hours later)				
VII-'36	25	Glucose	697	+ 188
VIII	50	Fructose	151	— 50
IX	50	Sucrose	347	— 203

#### COMPARATIVE COMBUSTIBILITY OF SUGARS

When the data for changes in blood sugar, in average non-protein respiratory quotients, in level of blood ketones and total ketosis (see below) are brought together, the results are as shown in table 7. The changes for blood sugar and ketonemia are calculated from determinations made just before the first dose of sugar and at 3 hours (average 3.3 hours) following its ingestion. The respiratory metabolism periods from which change in non-protein quotients are calculated, in all cases, immediately preceded taking of blood and urine samples, so that they fairly indicate the mixture of non-protein foodstuffs being oxidized over 3 or more hours leading up to the blood conditions found. Fat in large quantity was present

in the alimentary tract (see tables 1 to 4) while the first quotients were being obtained and fat plus sugar for the later ones.

A graphic method is not required to show the absence of parallelism either between blood sugar change and change in ketonemia or between change in the character of combustion and effect on ketosis. In all cases but one (VII-'36) blood sugar was accumulating during the 3 hours following the

TABLE 7  
*Combustibility of sugars in relation to ketosis*

SUBJECT NO.	AMOUNT	SUGAR	CHANGE IN LEVEL BL. SUGAR	CHANGE IN CO <sub>2</sub> COMBINING POWER	CHANGE IN KETO-NEMIA	CHANGE IN NON-PROTEIN R.Q.	CHANGE IN TOTAL KETOSIS
First dose							
VII-'35	25	Sucrose	mg. % + 5	vol. %	mg. % - 6.7	+ 0.10	gm. - 0.53
VII-'36	25	Glucose	- 35	- 4.4	+ 1.9	- 0.02	+ 0.16
IX	50	Sucrose	+ 53	+ 6.0	- 21.6	+ 0.01	- 1.37
I	50	Glucose	+ 54		+ 4.9	- 0.01	+ 0.42
VIII	50	Fructose	+ 22	- 0.5	- 6.6	+ 0.23 <sup>1</sup>	- 0.41
II	100	Sucrose	+ 105		- 6.8	+ 0.12	- 0.45
IV	100	Glucose	+ 48		- 12.4	+ 0.08	- 0.96
VI	100	Sucrose	+ 95		- 11.7	- 0.04	- 0.72
V	100	Glucose	+ 32		- 16.0	+ 0.01 <sup>1</sup>	- 1.03
Second dose (18 hours later)							
VII-'36	25	Glucose	+ 13	- 3.7	- 4.9	+ 0.01	
VIII	50	Fructose	- 15	+ 5.8	- 9.0	+ 0.199 <sup>1</sup>	
IX	59	Sucrose	+ 15	+ 5.0	- 25.8	- 0.03	

<sup>1</sup> This subject exhibited hyperpnoea.

ingestion, proving that absorption was going on rapidly. The peak of the absorption curve naturally must have come before the 3.3 hours (average) collection of blood. The figures show not the extent of absorption, but rather the amount of sugar remaining in circulation at this time. The level remaining is not proportional to the amount given but reflects rather the tolerance, meaning capacity for removal of blood sugar. In four cases (VII-'35, VIII,<sup>3</sup> II, IV) the rise in R.Q. proves

<sup>3</sup> This subject only exhibited definite hyperpnoea probably accounting in some measure for the rise in R.Q., though it was present in both series of respiratory determinations.

that combustion of sugar must have been taking place tending to reduce the blood sugar. In all these tests (two after sucrose, one after fructose and one after glucose) the ketonemia at the same time was falling. The rise in R.Q. therefore was not influenced by greater ketosis and consequently greater release of  $\text{CO}_2$  through the lungs (see discussion of  $\text{CO}_2$  combining power below). In only one other case (VI) is the change in R.Q. significant and in this instance it fell. Without significant change in carbohydrate combustion, as the remaining subjects show, ketosis may rise slightly or fall considerably under the influence of sugar. The only other way of disposing of carbohydrate (aside from excretion, which never amounted to more than 1 or 2 gm., or fat formation which could scarcely take place without a significant rise in R.Q.) is glycogen formation. Including effects of the second dose of sugar (bottom of table 7) there are five instances of definite reduction of ketonemia within 3.3 hours without evidence of concomitant combustion of sugar. Three of these occur following sucrose and two following glucose. There are two instances of slight increase in ketonemia without evidence of combustion. If we assume that most of the effect to reduce ketonemia occurs in the liver (p. 647) where ketogenesis itself is believed mainly to take place, perhaps the last two cases, both with smaller doses of glucose, may be explained as a preliminary displacement of ketone substances from the liver.

It is unfortunate that the  $\text{CO}_2$  combining power of the blood plasma was not determined in all the experiments. In three of the instances where it was determined a definite rise in this property coincided with a considerable reduction of the ketonemia, as would be expected. In two instances where the ketonemia changed but little (a rise of 1.9 and a fall of 4.9 mg.%) there was a concomitant small decrease in combining power, one accompanied by a fall of blood sugar, the other by a small rise. With at least two others showing decrease in ketonemia and increase in respiratory quotient, it seems probable that a rise in  $\text{CO}_2$ -combining power must have occurred even though it was not determined.



The last column of table 7 contains the term 'total ketosis.' By this term is meant the total amount of ketone substances expressed as acetone contained in the entire circulation. This value is found by multiplying the blood volume taken as 8.8% of the body weight in kilograms (Keith, Rowntree and Geraghty, '15; Seyderhelm and Lampe, '25) by the ketonemia in milligrams per cent. The change in total ketosis naturally bears the same sign as the change in milligrams per cent. But in comparing different subjects the two values are not in the same proportion on account of differences in weight. Change in total ketosis, however, bears no closer relationship to changes in combustion or to changes of blood sugar levels.

Concluding this section, it may be stated emphatically that reduction of ketosis does not appear to depend upon combustion of sugar.

#### RATE OF KETOGENESIS AND CARBOHYDRATE COMBUSTION

The concept of rate of ketogenesis has been sufficiently explained (p. 646). It was devised as an expression of total ketone production as measured by change in total ketosis and in ketonuria in relation to time. A positive value may mean that ketone substances are being accumulated in the body in addition to being excreted, or falling less rapidly in the body than being excreted. A negative value means decrease in the body more rapidly than they are being excreted. Ketone substances then are being disposed of in some other way (presumably by oxidation).

The data for comparison of change of rate in ketogenesis with changes of carbohydrate combustion are shown in table 8. Sucrose invariably caused a decreased rate. Glucose in small quantity (25- and 50-gm. doses) caused an increased rate; but in larger quantity (100 gm.) caused twice as great a decreased rate as the same quantity of sucrose. All these effects were measured to 3 hours (average 3.3 hours) after ingestion. Prolonged over a greater period (8 hours) glucose reduces ketogenesis in the phlorhizinized dog (Murlin and Manly, '36) more than sucrose or fructose. Quite probably this would

be true also in these human subjects, for in three of them who took glucose the ketonuria fell much farther over night between the fourth and fifth days of the diet, than it did in three who took sucrose. The ketonemia fell less for glucose. The ingestions of fat, however, were not equalized and the collections of blood and urine were not carried out in such a

TABLE 8  
*Comparison of rate of ketogenesis with carbohydrate combustion*

SUBJECT	FAT OR SUGAR	RATE OF KETOGENESIS	CHANGE IN RATE OF KETOGENESIS	CHANGE IN NON-PROTEIN R.Q.	CHANGE IN CARBOHYDRATE METABOLISM
		<i>mg./hr.</i>	<i>mg./hr.</i>		<i>Cal./hr.</i>
VII-'35	152 gm. fat	148			
	25 gm. sucrose	— 20	— 168	+ 0.10	+ 26.0
VII-'36	140 gm. fat	171			
	25 gm. glucose	245	+ 74	— 0.02	— 2.5
IX	123 gm. fat	637			
	50 gm. sucrose	— 192	— 829	+ 0.01	— 0.35
I	171 gm. fat	337			
	50 gm. glucose	617	+ 280	— 0.01	— 1.10
VIII	132 gm. fat	490			
	50 gm. fructose	93	— 397	+ 0.23 <sup>a</sup>	+ 44.7 <sup>a</sup>
II	91 gm. fat	227			
	100 gm. sucrose	10	— 217	+ 0.12	+ 31.0
IV	95 gm. fat	661			
	100 gm. glucose	83	— 517	+ 0.08	+ 4.3
VI	152 gm. fat	146			
	100 gm. sucrose	— 102	— 248	— 0.04	— 11.0
V	155 gm. fat	366			
	100 gm. glucose	— 67	— 433	+ 0.01	+ 2.8

<sup>a</sup> This rise of R.Q. and carbohydrate calories is questionable because of hyperpnoea; the only subject presenting this complication.

way as to make possible the calculation of the rate of ketogenesis for the night period.

Subjects II, IV, V and VI in this table fall into two paired experiments. The first two were people of comparatively low tolerance for fat; the latter two of comparatively high tolerance (tables 1 and 2). They were fairly well-paired also for weight. Approximately the same meal of butter fat in

each pair was followed at the same interval by sucrose in one member and glucose in the other. The rate of ketogenesis was changed by sucrose, in the two receiving that sugar, to nearly the same extent. The comparison for glucose is not quite so close; but in each pair its effect is unmistakably greater than that of sucrose—mean for the two sugars — 425 and — 232 mg. per hour, respectively.

Change in average R.Q. and in carbohydrate combustion, expressed as Calories per hour, from three periods just preceding and just following the first dose of sugar (tables 1 to 7, preceding paper) are shown in the last columns of table 8. It is evident at a glance that no parallelism between either of these changes and the change in ketogenesis exists. Increase in carbohydrate combustion occurs with decreased ketogenesis in four subjects (excluding VIII) and the reverse occurs in two (VII-'36 and I). Thus far and no farther do the results fit into the theory of Shaffer for ketogenesis-antiketogenesis. The respiratory quotients and the determination of ketone substances were as accurate, we believe, as it is possible to make them. Signs of 'blowing off' CO<sub>2</sub> were present in only one subject.<sup>4</sup>

In detail the quantitative relationship falls down completely. For example, the person (subject IX) exhibiting the greatest reduction of ketogenesis showed only a negligible change of carbohydrate metabolism. Other detailed comparisons in the table show no proportionality between the changes of rate of ketogenesis and antiketogenesis.

#### EFFECTS OF SUGAR GIVEN 'EARLY' AND 'LATE'

There remain to be compared the effects on the rate of ketogenesis in comparison with the extra carbohydrate combustion caused by the same dose of the same sugar administered 'early' and 'late' after the last fat was taken (table 9).

\*Subject VIII who exhibited such signs is excluded, although with definite hyperpnoea he fits the theory; and for subject VII-'36, who exhibited hyperpnoea in two periods after fat on June 8th (p. 632 of preceding paper), only the third period was used.

Only subject II exhibits anything like a proportionality between the total ketosis, and the amount of carbohydrate oxidized, and none shows any proportionality between change of ketogenesis and of carbohydrate combustion. Nearly the same extra oxidation per hour following the ingestion of 100 gm. sucrose 'early' and 'late' by subject II accompanied a like reduction in absolute level of ketones in the body. But from the ketogenesis figures it is evident that this result is due to a disappearance of ketones amounting to 142 mg. per

TABLE 9  
*Comparative effects of sugar given early (E) and late (L) after fat meal*

SUBJECT	SUGAR	CHANGE IN TOTAL KETOSIS	CHANGE IN RATE OF KETOGENESIS	CHANGE IN CARBOHYDRATE METABOLISM
		<i>gm.</i>	<i>mg./hr.</i>	<i>Cal./hr.</i>
I	50 gm. glucose	E + 0.42	+ 617	- 1.1
	50 gm. glucose	L - 0.35	- 46	+ 16.1
II	100 gm. sucrose	E - 0.45	+ 10	+ 30.6
	100 gm. sucrose	L - 0.42	- 142	+ 33.6
IV	100 gm. sucrose	E - 0.96	+ 83	+ 4.3
	100 gm. sucrose	L - 2.31	- 738	+ 23.3
V	100 gm. glucose	E - 1.03	- 67	+ 2.8
	100 gm. glucose	L - 0.21	- 52	+ 30.2
VI	100 gm. glucose	E - 0.72	- 102	- 10.9
	100 gm. glucose	L - 0.14	- 18	+ 28.7
VII	25 gm. sucrose	E - 0.54	- 20	+ 25.8
	25 gm. sucrose	L - 0.40	- 80	+ 5.8

hour following the 'late' dose, as compared with an increased formation of 10 mg. per hour following the 'early' dose. For subject I it can be said that the oxidation of 16 Cal. per hour from carbohydrate accompanied a disappearance of 46 mg. of ketones per hour. This is the effect of late administration; but a slight reduction in carbohydrate calories after the first administration was accompanied by a piling up of 617 mg. per hour. For subject IV slight oxidation 'early' accompanied reduction in level but the excretion proved that there was still a considerable ketogenesis. Five times as much oxidation 'late' accompanied more than twice as much

accumulation, in spite of the disappearance of nearly  $\frac{3}{4}$  of a gram ketones per hour. For the remaining three subjects greater disappearance (negative ketogenesis) goes along with lesser oxidation, while in each case lesser reduction of level goes with greater oxidation.

In every subject except VII oxidation of sugar takes place more readily when the fat is out of the way ( $11\frac{1}{2}$  to 15 hours after last meal). In three of the five this greater oxidation is accompanied by greater disappearance of ketones; in the other two just the reverse. Including subject VII we have exactly reverse effects in half the subjects. The favorable effects therefore cannot be ascribed to oxidation of sugar. Furthermore, since 50 gm. glucose with subject I produced nearly as much disappearance of ketones (late) as did 100 gm. in subject V; and since 25 gm. sucrose in subject VII produced five times as much disappearance (late) as did 100 gm. of the same sugar in the same time in subject VI (notwithstanding abundant evidence of their absorption into the blood) we must conclude that there is no other stoichiometric relationship between sugar and fatty acids in relation to ketolysis, under these conditions.

#### DISCUSSION

It would not be profitable at this time to calculate by Shaffer's first ('21 b) or second ('22) method the K: A ratios, because his fat factors are derived from an arbitrary mixture of the higher fatty acids found in human food and the human body, whereas the fat fed in these experiments was of a wholly different composition. Butter fat from cows fed on normal rations according to the most reliable data (Holland, Garvey, Pierce et al., '23) has the following composition of fatty acids, from which the composition of the neutral fat, may be readily calculated (Holland, Reed and Buckley, '15).

	<i>Fatty acid</i> %	<i>Neutral fat</i> %
Butyric acid	2.93	3.71
Caproic acid	1.90	2.29
Caprylic acid	0.79	0.90
Capric acid	1.57	1.70
Lauric acid	5.85	6.31
Myristic acid	18.78	21.00
Palmitic acid	15.17	15.89
Stearic acid	14.91	15.44
Oleic acid	31.89	32.85
	<hr/>	<hr/>
Total	94.79	100.09

This distribution would give an average neutral fat of the formula  $C_{49.3}H_{92.6}O_6$ . The triglyceride would have the composition  $C_3H_5(C_{15.4}H_{29.2}O_2)_3$  which on complete oxidation would yield an R.Q. of just 0.71 and the FA:G R.Q. i.e., after conversion of all the glycerol to glucose (Shaffer, '21 c), would be 0.688. However, there is no certainty that the nine different fatty acids would be absorbed in the same proportion in which they are present in cream, particularly in a 3- to 5-hour period such as has been considered in this paper, or that they would reach the tissues in this proportion one to another. Such a calculation must wait for more reliable data on differential absorption. Hughes and Wimmer ('35) have shown that the water soluble fatty acids, especially butyric and caproic, must in all probability be absorbed by the portal system and that as a consequence they probably escape the glyceride synthesis. The more rapid passage of these lower fatty acids to the liver naturally would facilitate their transformation to ketone bodies as compared with fatty acids which must travel by the thoracic duct and then be broken down farther to form ketone substances.

A more important matter is the effect of the ketolytic action of sugar on the actual as compared with the theoretical change in respiratory metabolism. Lusk ('28, p. 671) has shown that the formation of 36 gm.  $\beta$ -hydroxybutyric acid from 100 gm. neutral fat would reduce the R.Q. from 0.707 to 0.669. But if this amount of acid were to neutralize  $NaHCO_3$ , sufficient  $CO_2$  would be released to restore the R.Q. to 0.715. Reversing this

process by combustion of  $\beta$ -hydroxybutyric acid, Na would be released which then would bind up  $\text{CO}_2$  causing a reduction of the R.Q. According to the hypothesis of Shaffer ('22), this combustion would require the simultaneous combustion of sugar (glucose or fructose) in the proportion of 1 mol. to 2 of aceto-acetic acid.

The maximum effect of sugar to reduce ketogenesis in this work is shown in table 8, where with subject IX 50 gm. sucrose caused a change of — 0.829 gm. per hour expressed as acetone. Expressed as aceto-acetic acid the amount would be 1.32 gm. per hour. Oxidation of this and the oxidation of  $\frac{1}{2}$  mol. of glucose or fructose per mol. of aceto-acetic would produce the following effect on the pre-sugar metabolism of this subject June 23, 1936, table 7, preceding paper).

	$\text{CO}_2$ , L./hr.	$\text{O}_2$ , L./hr.	R.Q.
Average respiratory exchange			
before sugar	13.98	19.70	0.71
Oxidation of 1.32 gm. aceto-acetic	1.28	1.28	
	<hr/>	<hr/>	
	15.26	20.98	0.73
Oxidation of 1.29 gm. glucose	0.96	0.96	
	<hr/>	<hr/>	
	16.22	21.94	0.74
Subtract $\text{CO}_2$ bound by Na, set free			
from 1.32 gm. aceto-acetic	0.32		
	<hr/>	<hr/>	
	15.90	21.94	0.725

The actual net change in R.Q. in this case (table 8), corresponds very closely to the theoretical, but it is the only one which does. It may be objected that the change of R.Q. caused by the elimination of even a maximum ketosis is so small that it might well be obscured in the total respiratory exchange. This can scarcely be an answer to the diverse relations between change of ketogenesis brought about by carbohydrate and the rate of carbohydrate metabolism disclosed by this study. There must be some other function served by carbohydrate in ridding the body of ketone substances. It may be only preparatory to the kindling action.

There is growing evidence that this function is glycogen formation in the liver. Thannhauser ('29) has reviewed it to 1929. His own conviction is contained in the statement, "we see as well in experimental pancreatic diabetes as in phlorhizin diabetes and in severe human diabetes that glycogen impoverishment of the liver and ketonuria are very intimately related" ('29, p. 311). Of the newer evidence mention may be made especially of the work of Steppuhn and Timofejewa ('30) who find a close relationship between the ketosis and the increased protein metabolism of diabetes brought about through activation of proteolytic enzymes in the liver by the acidification of the ketone substances. They picture the sequence of events as follows: depletion of glycogen and mobilization of peripheral fat into the liver and formation of ketones→production of local decrease of pH in the liver→increased activity of proteolytic enzymes and protein destruction formation of glycogen from protein→removal of ketones. "We hold to the fact," they say, "and the whole literature supports the view, that the ketone level is . . . an excellent glycogenometer of the liver" (*loc.cit.*, p. 471). The recent work of Shapiro ('35) in Deuel's laboratory directs attention to the parallelism between ketolytic action and glycogen forming capacity in rats of glucose, l (+)-lactic acid, pyruvic acid and glycerol. In striking confirmation of the ideas of Steppuhn and Timofejewa was the ketolytic effect of ethyl alcohol, accounted for by an increased catabolism of protein.

#### SUMMARY

1. In connection with the determination of specific dynamic action of butter fat and of superimposed sugar reported in the preceding paper, the urines collected for nitrogen analysis and blood samples, appropriately spaced, were analyzed for the ketone substances and sugar in eight of the nine subjects. It thus was possible to compare ketogenesis on the high fat diet with the respiratory metabolism and changes induced in both by the administration of sugar (C.P. corn glucose, fructose and sucrose).



2. Analysis, like the calorimetric observations, began on the third day of the all-cream diet. The increase in ketonemia from the third to the fourth day was 8.8 mg.%, or 33% greater. The increase in ketonuria was 41%. A meal of fat containing from 240 to 450 gm. 4X cream given on the fourth day increased the ketonemia in an average period of 4.1 hours 22%; the average increased excretion in the same time was 29%.

3. Sugar was administered at two intervals after the fat, 3 to 5 hours, called 'early' and 11 to 15 hours, called 'late.' Doses varied from 25 to 100 gm. The average effect of the 'early' dose on blood ketones in 3 hours (average 3.3 hours) was a reduction of 8.3 mg.%—the range from +4.9 mg.% to -21.6 mg.%. The average reduction with sucrose was 10.7 mg.% and with glucose 5.4 mg.%. There was no definite relation of the effect to the amount of sugar given. Excretion was sometimes reduced and sometimes increased.

4. Blood sugar increased in all cases but at the 3.3 hour sampling varied from -35 mg.% to +105 mg.% from the level before sugar ingestion. No parallelism was shown between blood sugar level and change in ketonemia or in total ketosis (meaning mg.% in blood multiplied by blood volume, taken as 8.8% of body weight).

5. Change in rate of ketogenesis, calculated from the total ketosis and the ketonuria, in milligrams per hour varied from +280 mg. to -828 mg. Sucrose invariably caused a decrease, while glucose in smaller doses (25 and 50 gm.) caused an increase and in larger doses (100 gm.) a decrease. In two paired experiments (100 gm. of sugar following nearly equal feedings of cream, the two subjects in each comparison being nearly equal in weight) glucose caused a decrease twice as great as sucrose.

6. The increased combustion of carbohydrate in five experiments was more than sufficient to extinguish the ketogenesis according to Shaffer's theory, but in no case did so within 3 to 4 hours. In one experiment out of nine the actual change agreed with theory. In the remaining three there was a decrease in carbohydrate oxidized. The CO<sub>2</sub>-combining power

of the blood increased in two experiments with sucrose and one with fructose; it decreased in two with glucose and one with fructose. The R.Q. changes found apparently were not affected by changes in  $\text{CO}_2$ -combining power except in one instance with fructose.

7. In comparing the 'late' dose of sugar with the 'early' it was found that the change in rate of ketogenesis was negative following the 'late' dose in all of the six experiments, whereas the 'early' dose caused a positive change in three of the six. In all but one experiment there was a greater combustion of sugar following the 'late' dose than following the 'early.'

#### CONCLUSIONS

1. Sugar causes a marked reduction in total ketosis within 3 to 4 hours.

2. Sucrose is somewhat more effective in doses up to 50 gm.; glucose more effective in 100 gm. doses. Fructose stands between the other two.

3. None of the sugars completely extinguished the ketogenesis in 3 to 4 hours, although the increase in carbohydrate combustion was more than sufficient, according to Shaffer's theory. In one instance out of nine the change in ketogenesis exactly agreed with the increased combustion, according to theory on the basis of 1 mol. glucose to 2 mols, aceto-acetic acid.

4. A 'late' dose of sugar following an 'early' dose was much more effective than the 'early' one in reducing ketogenesis. Also it increased carbohydrate combustion more. This is interpreted as due to replenishment of glycogen in the liver, possibly because of insulin production stimulated by the early dose.

5. Glycogen formation seems to be as important as combustion in the antiketogenic action of sugar. Quite possibly the presence of a certain minimal 'normal' content of glycogen in the liver is a condition precedent to the normal oxidation of fatty acids. There is somewhat more evidence, however, that this normal content of glycogen prevents the formation of ketone substances in the liver.

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# THE AVAILABILITY OF *d*(—)-LYSINE FOR GROWTH <sup>1</sup>

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ONE FIGURE

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Several years ago, in a study of the replaceability of lysine in the diet by related hydroxy compounds, McGinty, Lewis and Marvel ('24-'25) noted that natural *l*(+)-lysine promoted somewhat better growth than did synthetic *dl*-lysine. Recently we have verified this observation in an extensive series of tests (Berg and Dalton, '34) which suggested that the unnatural *d*(—)-lysine half of the *dl* mixture could probably not be utilized at all by the rat for growth purposes. For direct proof of this possibility we undertook the resolution of *dl*-lysine (Berg, '36) and are now presenting data obtained on a series of animals fed lysine-deficient diets supplemented with natural *l*(+)-lysine or with its optical *d*(—) antipode.

## EXPERIMENTAL

The lysine-deficient diet employed contained zein 19.5, tryptophane 0.2, cystine 0.2, histidine monohydrochloride 0.1, sucrose 15.0, starch 34.5, hydrogenated vegetable oil<sup>2</sup> 19.0, cod liver oil 5.0, salt mixture (Hawk and Oser, '31) 4.5, and agar 2.0%. This was supplemented by feeding two 100 mg. 'vitamin-B complex' tablets (Harris) separately to each animal daily. Zein was used as the chief source of nitrogen because it is apparently entirely devoid of lysine. It was

<sup>1</sup> The designation of generic series by letter and direction of rotation by sign is in accordance with the system of Freudenberg (see Berg, '36).

<sup>2</sup> Crisco.

fed at a higher level than we have usually fed our proteins or protein hydrolysates because it has been shown to escape digestion in part (Mason and Palmer, '34). The use of tryptophane as a supplementing agent is, of course, necessary. To avoid the possibility of cystine or histidine deficiencies, these amino acids also were supplied. Histidine addition was later found unnecessary and was therefore subsequently omitted. The vitamin B complex was used instead of yeast to avoid unnecessary addition of extraneous protein. The zein was prepared by extracting corn gluten with 75% alcohol, precipitating with water, redissolving in 70% alcohol, filtering, concentrating, and reprecipitating with acetone, essentially as directed by Brazier ('30).

The diet was fed to a lot of twenty young rats from four litters for a period of 8 days, after which the animals were separated into seven groups of three each, except one which contained only two. Three of these groups now were given supplements of *d*(—)-lysine and three, supplements of the *l*(+) modification at levels of 0.5, 0.25 and 0.125%, respectively, replacing an equal quantity of zein. The diet of the remaining animals was left unchanged. The lysine was fed as the dihydrochloride; an equivalent amount of sodium bicarbonate was added to the diet to neutralize the hydrochloric acid thus included. The animals were housed in false bottomed cages and received food and distilled water ad libitum. The lysine isomers used were obtained by resolution of *dl*-lysine. The method of their preparation and their melting points, N content, and optical rotations have already been recorded (Berg, '36). After 20 days on the above diets, the histidine monohydrochloride was omitted in all cases, control animals having shown that its inclusion resulted in no growth acceleration. After 80 days, the lysine supplement was omitted from the diets of the animals of the *l*(+) series; the rats fed the *d*(—) supplements and the two remaining animals which had received no supplement were fed 0.5% of *l*(+)-lysine. Twelve days later the experiment was terminated.

## DISCUSSION

The results are summarized in figure 1 and table 1. In every case the rats on the  $l(+)$ -lysine supplements showed definite growth responses. On the other hand, no difference

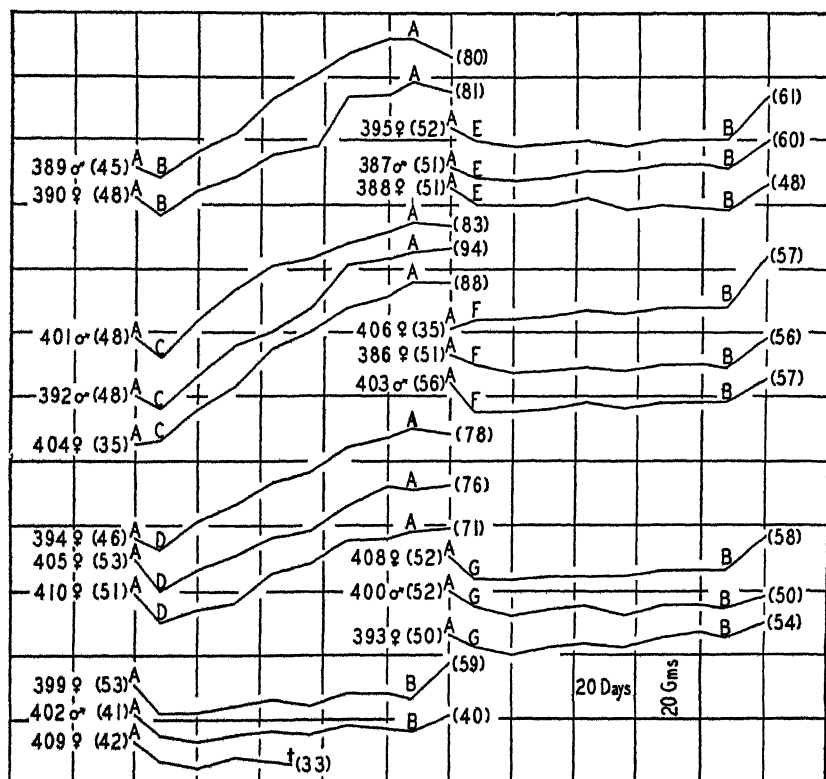


Fig. 1 Growth on  $l(+)$ - and  $d(-)$ -lysine. Initial and final weights of the rats are given in parentheses. A represents the unsupplemented lysine-deficient diet. B, C and D indicate similar diets containing 0.5, 0.25 and 0.125%  $l(+)$ -lysine supplements, respectively; E, F and G, diets containing corresponding supplements, respectively, of  $d(-)$ -lysine. The dagger indicates the death of rat 409.

was detectable, either in growth or behavior, between the rats fed  $d(-)$ -lysine and those receiving no lysine supplement. In general, animals which showed a definite growth response also showed a greater food consumption. The preliminary and after periods show that the lack of added  $l(+)$ -lysine in

the diet either results in loss of weight or permits extremely slow growth; addition of *l*(+)-lysine to the diet of the rats which had previously grown only very slowly, if at all, caused a definite growth response, thus indicating that the 88-day plateau was the result only of an unfavorable dietary regimen.

TABLE 1  
*Food consumption*

DAYS	RAT NO. AND SEX	AVERAGE DAILY FOOD CON- SUMPTION	RAT NO. AND SEX	AVERAGE DAILY FOOD CON- SUMPTION	RAT NO. AND SEX	AVERAGE DAILY FOOD CON- SUMPTION	LYSINE SUPPLEMENT
		<i>gm.</i>		<i>gm.</i>		<i>gm.</i>	%
1- 8	389♂	3.5	390♀	3.3			0.0
9- 88		4.2		4.3			0.5 <i>l</i> (+)
89-100		3.3		4.7			0.0
1- 8	387♂	3.8	388♀	2.9	395♀	4.0	0.0
9- 88		3.4		2.5		3.1	0.5 <i>d</i> (—)
89-100		3.5		2.3		4.3	0.5 <i>l</i> (+)
1- 8	392♂	3.0	401♂	3.1	404♀	3.3	0.0
9- 88		4.0		4.6		4.5	0.25 <i>l</i> (+)
89-100		3.0		3.7		3.8	0.0
1- 8	386♀	3.5	403♂	3.3	406♀	3.1	0.0
9- 88		3.4		2.7		2.9	0.25 <i>d</i> (—)
89-100		3.2		3.2		4.0	0.5 <i>l</i> (+)
1- 8	394♀	3.5	405♀	3.4	410♀	3.0	0.0
9- 88		4.6		4.7		3.7	0.0125 <i>l</i> (+)
89-100		3.8		4.1		3.5	0.0
1- 8	393♀	4.0	400♂	3.6	408♀	4.0	0.0
9- 88		3.3		3.0		3.3	0.125 <i>d</i> (—)
89-100		3.3		2.9		3.5	0.5 <i>l</i> (+)
1- 8	399♀	3.8	402♂	2.5	409♀	3.5	0.0
9- 88		2.9		2.1		2.9 <sup>1</sup>	0.0
89-100		3.1		2.0			0.5 <i>l</i> (+)

<sup>1</sup> Nine to 50 days.

In the present series of studies the animals on 0.5% and 0.25% *l*(+)-lysine supplements grew at very similar rates; in the earlier studies (Berg and Dalton, '34) the weight increment on the higher lysine supplement was appreciably greater.



Corresponding discrepancies in food consumption in the two series were also observed. The same lot of zein was used in both studies. In the first series it was fed at a 15% level, in the second at 20%. The only other significant difference in regimen was the nature of the vitamin B complex. In the earlier series this was supplied in the form of yeast, in the later as yeast concentrate.

In any event, however, the data indicate clearly that the optical antipode of natural *l*(+)-lysine cannot be utilized for growth.<sup>3</sup> In this respect lysine is comparable to cystine (du Vigneaud, Dorfmann and Loring, '32), but different from tryptophane (du Vigneaud, Sealock and Van Etten, '32; Berg, '34), histidine (Cox and Berg, '34), and methionine (Jackson and Block, '33).

#### CONCLUSION

*d*(—)-lysine is unable to promote growth when fed as a supplement in a lysine-deficient diet.

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<sup>3</sup> A personal communication from Dr. W. C. Rose indicates that he also has found that *d*(—)-lysine fails to support growth.

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